Synthesis and Biochemical Function of Selenocysteine-containing Peptides(Dissertation_全文)

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Introduction

Selenium belongs to the VIb group of the periodic table and possesses both metallic and non-metallic characteristics. The element lies between the non-metals, oxygen and sulfur and the metals, tellurium and polonium in the same group, and between bromine, a non-metal, and arsenic, a metal, in the same period in the table. However, physicochemical properties of selenium resembles more or less those of sulfur (Table I)(1).

Organoselenium compounds, in general, are less stable and more reactive than the corresponding sulfur analogs and these properties may account for the toxicity of selenium when it is incorporated indiscriminately in place of sulfur into cellular constituents. The characteristic properties of organoselenium compounds are as follows .

1. Organoselenium compounds are generally more reactive than the sulfur counterparts.
2. In contrast to thiols (pKa 8-9), selenols (pKa 5-6) are mostly ionized at neutral pH: selenols in enzymes are more acidic than thiols under physiological conditions.
3. Selenols are strong nucleophiles, but they also serve as good leaving groups.
4. Redox potential of selenol is generally lower than that of a corresponding thiol (e.g., selenocysteine vs. cysteine). This may explain the occurrence of selenols in redox enzymes such as formate dehydrogenase in strictly anaerobic bacteria.

Toxic effects of selenium were already recognized in the middle of 1930's, as an element that causes "alkali disease" (2) and "blind staggers" (3).

Table 1. Physiological properties of sulfur and selenium

Properties	S	Se	
Atomic Weight	32.06	78.96	
Natural Isotopes (%)	32 (95.1)	74 (0.87)	76 (9.02)
	33 (0.74)	77 (7.58)	78 (23.52)
	34 (4.18)	80 (49.82)	82 (9.19)
Covalent Radius (Å)	1.06	1.16	
Ionic Radius (Å) (X^-)	1.84	1.98	
Bond Energy (kJ / mol)			
X-X bond	212.9	184.5	
C-X bond	272	243	
pKa (RXH $RX^- + H^+$)			
R= H	6.94	3.88	
R= $CH_2CH(NH_2)COOH$	8.4	5.25	
Redox Potential (V)			
$H_2X(aq.)$ $X^0 + 2H^+ 2e^-$	0.23	0.30	
Solubility (mM, pH7.0, 25°C)			
$HXCH_2CH(NH_2)COOH$	0.38	2.35	

Nutritionists and physiologists were aware of only toxic and carcinogenic effects of selenium, and Pinsent (4) reported in 1954 that selenium is essential for production of formate dehydrogenase in *E. coli*. Then, nutritional studies established in the following years that selenium is an essential trace element for rat (5), birds (6, 7) and domestic animals (8, 9, 10). However, biochemical roles of selenium remained to be obscure until 1970's (11, 12).

In 1973, Rotruck *et al.*(13) and Flohe (14) showed that the mammalian

glutathione peroxidase contains a selenocysteine residue. Flohe proposed that the enzyme serves as a biochemical defense system against oxidative damage of cell constituents, and emphasized the physiological importance of the selenoenzyme. Concurrent studies on selenium deficiency, such as Keshan disease (15) and Kashin-Beck disease (16), also showed that selenium is an essential micronutrient.

Several selenoenzymes have been found in prokaryotic and eukaryotic organisms (Table II). Most of them contain selenium as a selenocysteine residue. Four redox enzymes, formate dehydrogenase, glycine reductase, hydrogenase and glutathione peroxidase contain selenocysteine residue at the active site.

Table II. Selenium-containing proteins

Enzyme	Selenium form	Origin	Reference
Glutathione peroxidase	SeCys	bovine erythrocyte	25
Selenoprotein P	SeCys	plasma	26
Tetraiodothyrosine 5'-deiodinase	unknown	thyroid	27
Glycine reductase	SeCys	*Clostridium sticklandii*	28
Formate dehydrogenase	SeCys	*Methanococcus vanielii*	29
	SeCys	*C. thermoaceticum*	30
	SeCys	*E. coli*	31
Xanthine dehydrogenase	unknown	*C. antidiurici*	32
	unknown	*C. cylindrosporum*	33
Nicotinate dehydrogenase	labile	*C. barkeri*	34
Thiolase	SeMet	*C. kluyveri*	35
Hydrogenase	SeCys	*M. vanielii*	36

The reaction mechanism and integral roles of the selenocysteine residue are best characterized for glutathione peroxidase (17). Low redox potential and high nucleophilicity of selenol are indispensable for the action of this selenoenzyme.

In 1984, a biologically active organoselenium compound, ebselene (called PZ51 at that time), was reported (18, 19). It became clear that many of the biological properties of ebselene were related with its glutathione peroxidase-like activity. A number of attempts were made to modify the structure of ebselene, and structure-active relationships of a series of anti-inflammatory benzisoselenazolones have been studied by Parnham *et al* .(20) and Tarino (21). Wilson *et al.*(22) and Cotgreave *et al.* (23) reported effects of some related compounds. The pharmacology of synthetic organic selenium compounds was recently reviewed by Parnham and Graf (24).

Glutaselenone is a selenium analog of glutathione disulfide, and contains selenocysteine residue. It (γ-glutamyl-selenocysteinylglycine) was synthesized chemically by a liquid phase method (Chapter I), and four diastereomers, the LL-, DL-, LD-, and DD-isomers, were produced.

I also describe synthesis of glutaselenone with γ-glutamyl transpeptidase (Chapter II). The glutathione peroxidase-like activity of gluataselenone diastereomers and its reaction mechanism (Chapter III), and synthesis of a novel selenocysteine-containing peptide which shows higher activity (Chapter IV). Biological effects of glutaselenone are also investigated (Chapter V).

Chapter I

Synthesis and Characterization of the Selenium Analogue of Glutathione Disulfide

Selenocysteine residue differs from its analogue, cysteine, in its redox potential and in various other chemical properties (18, 19, 37, 38), and occurs as an integral moiety in the active center of selenium-containing enzymes, such as mammalian glutathione peroxidase (39) and bacterial glycine reductase (28). Selenocysteine-containing glutathione analogues are expected to show interesting physiological functions, but their facile synthesis has not been described.

Glutathione is the most predominant endogenous thiol, and has many important biochemical functions in protein and DNA syntheses, amino acid transport, and protection of cells from oxidative damage (40, 41). The functions of glutathione are based on the high reactivity of the cysteine residue.

I here describe the synthesis of the selenium analogue of glutathione (i.e. γ-L-glutamyl-L-selenocysteinylglycine) and its characterization.

Materials and Methods

Materials.

L-β-Chloroalanine was synthesized from L-serine (42); disodium diselenide was from elemental selenium (43); L-selenocystine was from L-β-chloroalanine and disodium diselenide (44); and *Se*-(*p*-methoxybenzyl)-L-selenocysteine was from L-selenocystine, sodium borohydride and *p*-methoxybenzyl chloride

(45). Other reagents were of analytical grade.

N-(*p*-methoxybenzyloxycarbonyl)-*Se*-(*p*-methoxybenzyl)-L-selenocysteine (PMZ-SeCys(Se-PMBz)-OH).

Se-(*p*-Methoxybenzyl)-L-selenocysteine (4.0 g; 13.9 mmol) was dissolved in water (50 ml) containing triethylamine (4.2 ml) at 0°C. Then, *p*-methoxybenzyl azideformate (4.3 g) dissolved in dioxane (50 ml) was added to the solution. After 16 hr, the solvent was removed under reduced pressure, and the residue was dissolved in saturated citric acid solution (200 ml). The precipitate was collected by filtration, washed successively with 5% citric acid and water, and dried. The product was dissolved in minimum volume of ethyl acetate, then crystallized by addition of *n*-hexane (yield, 80%).

Benzyl *N*-(*p*-methoxybenzyloxycarbonyl)-*Se*-(*p*-methoxybenzyl)-L-selenocysteinyl-glycinate (PMZ-SeCys(Se-PMBz)-Gly-OBzl).

A solution of glycine benzyl ester *p*-toluenesulfonate (6.26 g) in dimethylformamide (40 ml) containing triethylamine (1.34 ml) was added to a solution of PMZ-SeCys(Se-PMBz)-OH (4.0 g; 9.2 mmol), *N,N*-dicyclohexyl-carbodiimide (2.0 g), 1-hydroxybenzotriazole (1.34 g) in dimethylformamide (40 ml) at 0°C. After 16 h, the solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (100 ml). After the mixture was washed successively with 5% citric acid, saturated sodium chloride solution, and 5% sodium bicarbonate, the ethyl acetate layer was dried over anhydrous magnesium sulfate, and evaporated. The product was dissolved in minimum amount of ethyl acetate, and was crystallized by addition of ethyl ether (yield: 83%).

Benzyl *N*-(*t*-butyloxycarbonyl)-(γ-benzyl)-γ-L-glutamyl-*Se*-(*p*-methoxybenzyl)-L-selenocysteinyl-glycinate.
(Protected glutaselenone)

PMZ-SeCys(Se-PMBz)-Gly-OBzl (1.8 g; 3.1 mmol) was treated with trifluoroacetic acid (6 ml) containing anisole (1 ml) at 0°C for 1 h. The oily residue obtained after evaporation was washed with *n*-hexane by decantation. The residue dried under reduced pressure was dissolved in dimethylformamide (20 ml), and reacted with α-benzyl-*N*-hydroxysuccimide ester-*N*-*t*-butyloxy-carbonyl-L-glutamic acid [prepared from *N*-*t*-butyloxycarbonyl-L-glutamic acid α-benzyl ester and 1-hydroxysucciimide (46)] (1.38 g) in the presence of triethylamine (0.42 ml). After 16 h, the solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (100 ml). After the mixture was washed successively with 5% citric acid, saturated sodium chloride solution, and 5% sodium bicarbonate, the ethyl acetate layer was dried with anhydrous magnesium sulfate. The solvent was evaporated, and the solid residue obtained was dissolved in minimum amount of ethyl acetate. The product was crystallized by addition of *n*-hexane (yield: 60%).

Deprotection of the protected glutaselenone.

A solution of the protected glutaselenone (1.35 g; 1.8 mmol) in a mixture of trifluoroacetic acid (52 ml), *m*-cresol (6 ml), trifluoromethanesulfonate (9 ml) and thioanisole (7 ml) was stirred at 0°C for 2 h. Trifluoroacetic acid was evaporated with flushed nitrogen gas. The residue was extracted with 20 ml of ethyl acetate and 20 ml of 3% ammonia solution in water. The water layer containing the diselenide form of glutaselenone was applied †to a Dowex 50Wx8 (H^+) column (1.0 X 25 cm). The column was washed with water until the eluate became neutral, and the fractions containing glutaselenone diselenide, which was eluted with 0.5% ammonia, were combined and

lyophilized to give a fluffy powder (0.13 g; yield, 20%).

Analytical methods.

Melting points were measured with a Yanaco micro melting point analyzer (Yanagimoto, Japan); optical rotations with an automatic polarimeter DIP-360 (Japan Spectroscopic Co.) at 25°C; absorption spectra with a Shimadzu MPS-2000 spectrophotometer; fluorescence spectra with a Hitachi MPF-4 fluorescence spectrophotometer at 20°C; circular dichroism with a spectro-polarimeter J-600 (Japan Spectroscopic Co.) at room temperature (about 20°C). Elemental analyses were done with a Yanaco CHN corder MT-3 (Yanagimoto). Glutaselenone diselenide was analyzed by high performance liquid chromatography (HPLC) with a COSMOSIL 5C18-AR column (4.6 X 150 mm, Nacalai Tesque, Kyoto, Japan), and detected at 220 nm. The mobile phase was 3.5% acetonitrile in water containing 0.1% trifluoroacetic acid (v/v) at a flow rate of 1.0 ml/min.

Results and Discussion

I have synthesized the selenium analog of glutathione disulfide by a liquid phase method, and named it glutaselenone diselenide. The *N*α-amino terminus and the side chain of amino acids were protected with acid-labile groups: *N*α-amino terminus, *t*-butyloxycarbonyl and *p*-methoxy-benzyloxycarbonyl group; and glutamic acid and glycine, a benzyl group. I have protected the selenol group of selenocysteine with *p*-methoxybenzyl group (47). Benzyl group has been previously used to protect the selenol group of selenocysteine in peptide synthesis. The *Se*-benzyl group is generally removed by the reduction with sodium in liquid ammonia, but side reactions occur (48, 49). In

contrast, the *p*-methoxy-benzyl group, which I used for protection of the selenol group of selenocysteine, was easily removed by acidolysis with trifluoromethanesulfonic acid. In order to protect the selenol group of selenocysteine residue from possible alkylation by *p*-methoxybenzyl-oxycarbonyl cation, I added thioanisole to trap the cation. Thus, *p*-methoxy-benzyl group can serve as an efficient protecting group for selenol in peptide synthesis, and can be used for synthesis of various selenocysteine-containing peptides. The overall yield of glutaselenone diselenide was about 9% based on the starting compound, *Se*-(*p*-methoxybenzyl)-L-selenocysteine.

The final preparation of glutaselenone diselenide was eluted as a single symmetrical peak at 11 min upon reverse phase HPLC under the conditions described under Materials and Methods: the sulfur counterpart glutathione disulfide, was eluted at 6 min under the same conditions.

The ^{1}H-NMR spectrum of the product was consistent with the structure of glutaselenone diselenide (Fig. 1). The proton signal of the β–methylene moiety of the selenocysteine appeared in a form of double multiplet at δ 3.2 and δ 3.5. Glutathione disulfide also showed a doublet at δ 3.0 and δ 3.3 as the proton signal of the corresponding position, whereas its reduced form, gave a single doublet at δ 3.0 as the proton signal of the same position.

Glutaselenone diselenide showed a broad absorption band between 270 and 400 nm (Fig. 2). An extinction coefficient of glutaselenone at 300 nm was calculated to be about 240. The spectrum is similar to that of selenocystine previously reported (49), and the broad absorption band is attributable to n-σ* transition of the diselenide bond (50). Glutaselenone diselenide showed a positive cotton band around 270 nm (positive) and 320 nm (negative) observed for L-selenocysteine are due to optically active transitions of the diselenide chromophore (51).

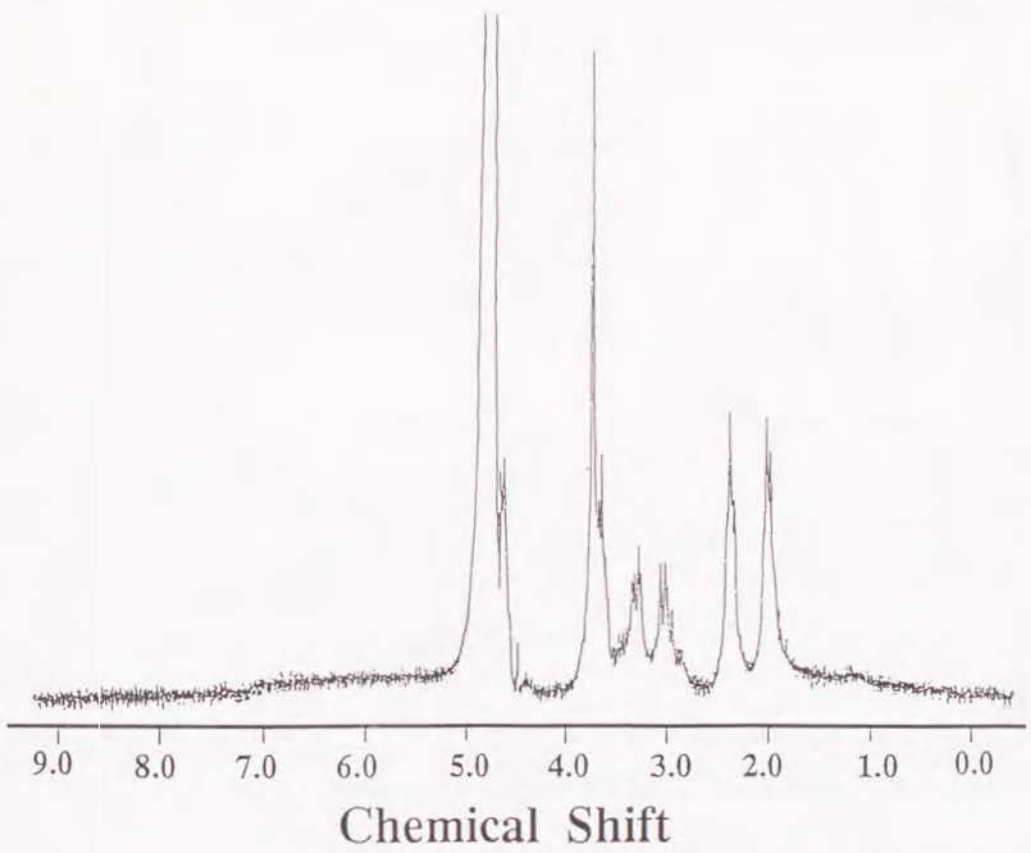

Fig. 1. ^{1}H-nmr spectrum of LL-glutaselenone (5 mg/500 µl D_2O).
δ1.9 (2H, m), γ-Glu-β–CH_2; δ2.4 (2H, m), γ-Glu-γ-CH_2; δ 3.1(2H, dm), Secys-β-CH_2; δ3.7 (1H, t), γ-Glu-α-CH; δ3.8 (2H, s), Gly-CH_2; α-Proton of selenocysteine residue was overlapping the peak of water at δ4.7.

The selenium analogues of several sulfur-containing peptides and proteins have been synthesized. The serine residue at the active center of subtilisin was replaced by selenocysteine residue by chemical modification (52). An analog of β-galactosidase whose methionine residues were extensively replaced by selenomethionine residue was obtained from a selenium-resistant mutant of *E. coli* grown on a medium containing sodium selenate (53). Selenocysteine analogs of oxytocin (54) and somatostatin (55) were synthesized by liquid

phase method.

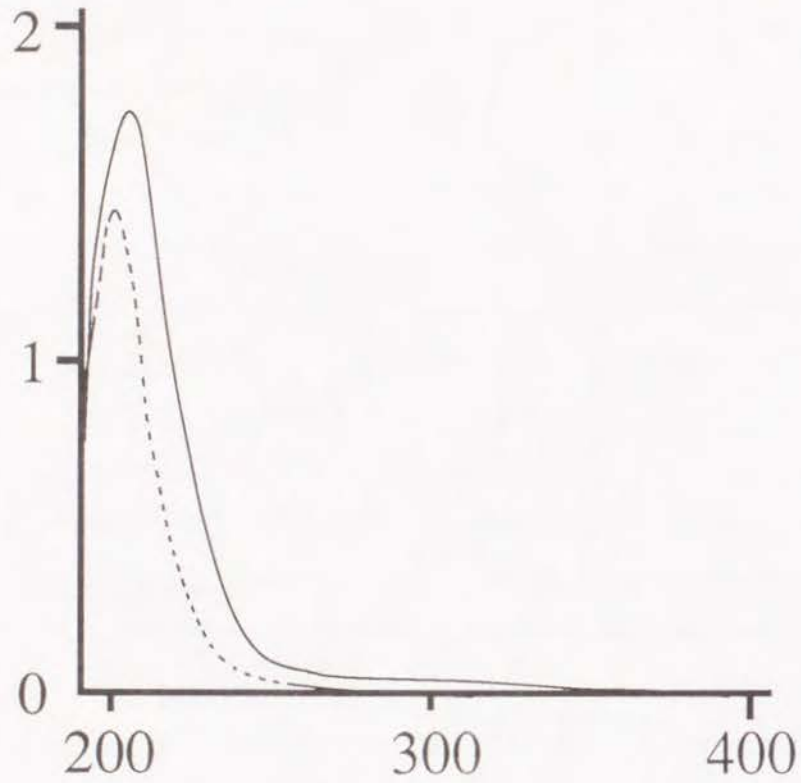

Fig. 2. Absorption spectrum of oxidized form of glutaselenone (—) and glutathione (- - -): solvent, water; concentration, 1 mM.

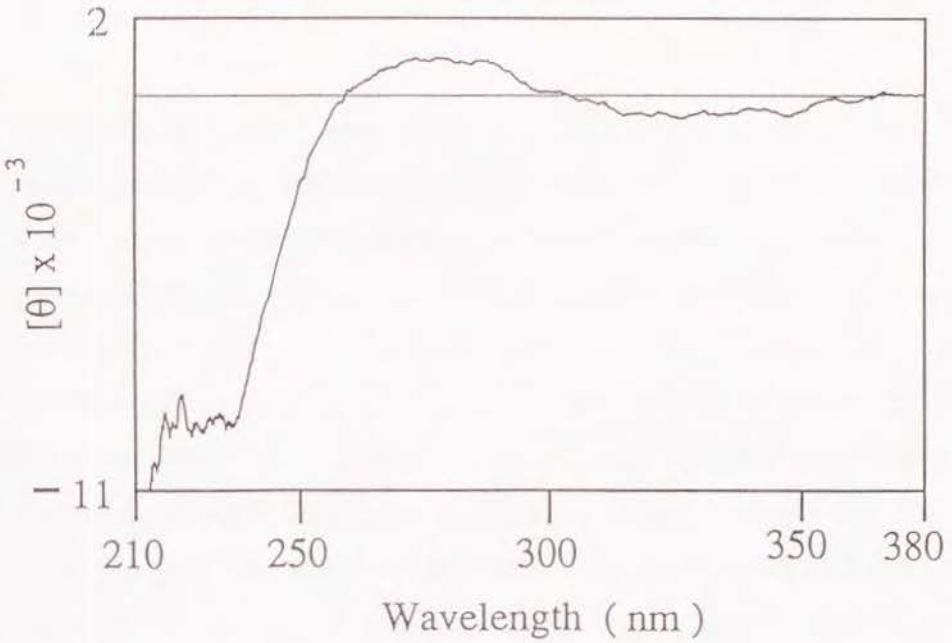

Fig. 3. Circular dichroism of oxidized form of glutaselenone in water.

I have synthesized the selenium analog of glutaselenone, and this is the first report of synthesis of a selenium-containing γ-glutamyl peptide.

Various physiologically-active peptides contain a disulfide bond as an essential moiety (56-58). The formation of disulfide bonds usually proceeds only slowly, and peptides containing disulfide bonds are synthesized with complicated techniques (59, 60). As compared with thiol, selenols are oxidized to form the diselenide much more efficiently (37). Thus, I propose to use selenocysteine instead of cysteine as analogues of physiologically-active peptides containing essential disulfide bonds. The selenocysteine analogs are expected to show a physiological function similar to the original peptide.

Summary

I synthesized the selenium analog of glutathione (i.e. γ-L-glutamyl-L-selenocysteinylglycine) by liquid phase method, and named it glutaselenone. The selenol of selenocysteine was protected by *p*-methoxybenzyl group, which was removed by acidolysis with trifluoroacetic acid in the presence of thioanisol. The overall yield of the final product, glutaselenone diselenide, was about 9% based on the starting compound, *Se*-(*p*-methoxybenzyl)-L-selenocysteine. Glutaselenone diselenide showed a broad absorption band between 270 and 400 nm and a positive circular dichroism band with a maximum around 270 nm, which were attributable to diselenide bond.

Chapter II

Enzymatic Synthesis of Glutaselenone with γ-Glutamyltranspeptidase

γ-Glutamyltranspeptidase (γ-GTP; EC 2.3.2.2) catalyzes the transfer of the γ-glutamyl (γ-glu) moiety of various γ-glutamyl amides to amino acids and peptides (28, 61, 62). It was used for synthesis of various γ-glutamyl compounds; γ-glu-DOPA (63-65), γ-glu-L-tyrosine (66) and γ-glu-*S*-benzyl-cysteinylglycine monomethyl ester (67).

Suzuki *et al.* cloned the *E. coli* K-12 *ggt* gene and developed a rapid purification method for the enzyme (68). The *E. coli* γ-GTP catalyzed γ-glutamyl transpeptidation from L-glutamine to various amino acids and peptides efficiently (69).

In this chapter, I describe a simple method for synthesis of glutaselenone with γ-GTP. The method is more advantageous to the chemical synthesis; L-glutamine is used as a γ-glutamyl donor in stead of protected form of L-glutamate, α-benzyl-γ-*N*-hydroxysuccimide ester-*N*-*t*-butyloxycarbonyl-L-glutamate.

Materials and Methods

Materials.

E. coli γ-GTP was kindly provided by Dr. H. Kumagai and Dr. H. Suzuki

(Kyoto University). Bovine kidney γ-GTP was purchased from Wako Pure Chemical Industries Ltd. A Simadzu MPS-2000 UV-VIS spectrophotometer was used to measure *A*410. Dimethylformamide (DMF), *N,N'*-dicyclohexyl-carbodiimide (DCC), 1-hydroxy-triazole (HOBT) were of analytical grade.

Synthesis of *Se*-Bzl-L-Secys-Gly-OMe.

Glycine methyl ester hydrochloride (0.33 g, 2.63 mmol) and triethylamine (0.36 ml) dissolved in DMF (5 ml) were added to a solution containing *N*-(*p*-methoxybenzyloxycarbonyl)-*Se*-benzyl-L-selenocysteine (1 g; 2.36 mmol), DCC (0.55 g), and HOBT (0.36 g) in DMF (10 ml) at 0°C. After stirred for 20 hours at room temperature, the mixture was filtered and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (100 ml) and washed successively with 5% citric acid (10 ml, twice), 5% sodium bicarbonate (10 ml, twice), and saturated sodium chloride solution (10 ml, twice). Ethyl acetate was removed by evaporation, and the residue was treated with 20 ml of 4 M HCl in ethyl acetate at room temperature for 2 hours. *Se*-Bzl-L-SeCys-Gly-OMe (0.73 g; 1.5 mmol) was crystallized from ether. ^{1}H-NMR (200MHz; D_2O): δ2.9 (2H, m, SeCys-β-CH_2), 3.7 (3H, s, Gly-OCH_3), 3.8 (2H, m, Se-CH_2-Ph), 4.0 (2H, s, Gly-CH_2), 4.1 (1H, m, SeCys-α–CH), and 7.3 (5H, s, Ph)

Assay of γ-GTP activity.

The activity of γ-GTP was determined as described previously for the *E. coli* γ-GTP (69). The assay mixture used for bovine kidney γ-GTP was supplemented with 10 mM magnesium dichloride.

Analysis and isolation of reaction products by HPLC.

Reaction products were analyzed and isolated with a Waters HPLC system equipped with a 600E system controller, a U6K universal liquid chromatograph

injector, a 484 tunable absorbance detector (wavelength fixed at 254 nm), and a Cosmosil 5C18 column (Nacalai Tesque Co.) (4.6 x 150 mm). The mobile phase was 11% acetonitrile/ 1.1% acetic acid/ 87.9% water (v/v/v) at a flow rate of 1.5 ml/min.

Results

Acceptor specificity of γ-GTP.

Both bovine kidney and *E. coli* γ-GTPs catalyzed transpeptidation reaction from γ-GpNA to various selenium- and sulfur-containing L-amino acids and dipeptides (Table III). D-Amino acids and L-cysteic acid were poor acceptors for both enzymes.

The two selenium containing acceptors, L-*Se*-Bzl-SeCys-Gly-OMe and L-*Se*-BzlOMe-SeCys-Gly-OMe, are substrates for glutaselenone synthesis. *E. coli* γ-GTP showed higher activity on these selenocysteine-containing peptides than the bovine kidney enzyme.

Donor specificity of γ-GTP.

γ-GTP utilizes various γ-glutamyl amides and peptides as γ-glutamyl donors; e.g., glutathione, γ-glutamyl-*p*-nitroanilide, and L-glutamine. Among these γ-glutamyl donors, L-glutamine is probably most convenient because of its high solubility in water and cheap availability. Activity of the bovine kidney and *E. coli* enzymes for L- and D-glutamine was examined.

Table III. Acceptor specificity of *E. coli* and bovine kidney γ-GTPs

Substrate	Relative rate (%)	
	E. coli γ–GTP	Bovine Kidney γ-GTP
Gly-Gly	100 (10.2 μM/min)	100 (4.4 μM/min)
L-Cys	52	72
D-Cys	0.0	3.2
L-Ser	1.6	30
L-Cysteic acid	0.0	0.0
L-Secys	49	41
D-Secys	0.0	0.0
L-*S*-Me-Cys	50	80
D-*S*-Me-Cys	0.0	1.9
L-*Se*-Me-Secys	50	79
L-*S*-Bzl-Cys-Gly-OMe	39	8.8
L-*Se*-Bzl-Secys-Gly-OMe	34	28
L-*Se*-BzlOMe-Secys-Gly-OMe	38	7.9
L-*Se*-Bzl-Secys-Gly-OMe	56	6.9
L-*Se*-Bzl-Secys-L-Ser-OMe	54	40
L-*Se*-Bzl-Secys-L-Thr-OMe	42	25
L-*Se*-Bzl-Secys-L-Tyr-OMe	18	42

γ-Glutamyl-*p*-nitroanilide was used as a γ–glutamyl donor (2.5 mM). The concentration of amino acids and peptides was 60 mM. Reaction was carried out at pH 8.7 at 37°C for 10 min. Difference in A_{410} between the reaction rates with or without an acceptor was used as a rate of transpeptidation reaction. Activity with Gly-Gly was expressed as 100%.

The bovine kidney enzyme catalyzed hydrolysis of L- and D-glutamine efficiently, but did not catalyze the transfer of glutamyl moiety from none of them (Table IV). Inefficient γ-glu transpeptidation from L- and D-glutamine was also

Table IV. Donor specificity of bovine kidney γ-GTP

Donor	Acceptor: Gly-Gly Hydrolyase Relative rate	Gly-Gly Transferase Relative rate	*Se*-Bzl-L-Secys-Gly-OMe Hydrolyase Relative rate	*Se*-Bzl-L-Secys-Gly-OMe Transferase Relative rate
γ-GpNA	100	100	100	100
(μM/min)	(7.2)	(8.8)	(10.6)	(2.0)
L-Gln	297	0	215	0
D-Gln	319	0	252	0
L-Asn	0	0	0	0

Table V. Donor specificity of *E.coli* γ-GTP

Donor	Acceptor: Gly-Gly Hydrolyase Relative rate	Gly-Gly Transferase Relative rate	*Se*-Bzl-L-SeCys-Gly-OMe Hydrolyase Relative rate	*Se*-Bzl-L-SeCys-Gly-OMe Transferase Relative rate
γ-GpNA	100	100	100	100
(μM/min)	(6.4)	(10.2)	(7.2)	(8.8)
L-Gln	107	131	109	91.4
D-Gln	198	127	133	409
L-Asn	0	0	0	0

Concentrations of γ-glutamyl donor and acceptor were 2.5 mM and 60 mM respectively. Reaction was carried out at pH 8.7 at 37°C for 10 min. γ-Glu-acceptor (transpeptidase activity) and glutamate (hydrolysis activity) formed were determined with an amino acid analyzer. Activity with γ-GpNA was expressed as 100%.

reported for rat kidney γ-GTP(70). The rat kidney γ-GTP acts on glutamine 530-fold less efficiently than on glutathione and L-γ-glutamyl-*p*-nitroanilide (γ-GpNA) (71). The *E. coli* enzyme catalyzed transfer of γ-glutamyl moiety of L- and D-glutamine to both Gly-Gly and *Se*-Bzl-SeCys-Gly-OMe efficiently

(Table V).

Therefore, *E. coli* enzyme was considered to be more useful than the bovine kidney enzyme for synthesis of L-*Se*-Bzl-glutaselenone monomethyl ester with L-glutamine and L-*Se*-Bzl-L-SeCys-Gly-OMe.

Effect of pH on transferase activity of γ-GTP.

Effects of pH on the enzymatic transpeptidation and hydrolysis were examined with γ-glutamyl-*p*-nitoanilide as a γ-glutamyl donor and *Se*-Bzl-L-SeCys-Gly-OMe or Gly-Gly (Fig. 4). *E. coli* γ-GTP catalyzed transfer of γ-glu to *Se*-Bzl-L-SeCys-Gly-OMe most efficiently at pH 9. For the bovine kidney enzyme, optimum pH for transpeptidation was also 9.

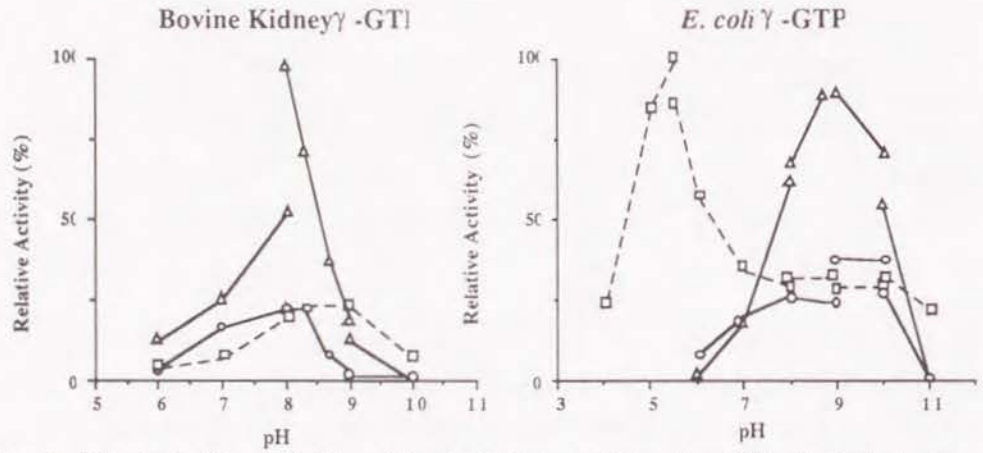

Fig. 4. Effect of pH on activities of bovine kidney and *E. coli* γ-GTP. Hydrolysis (-□-) and transpeptidation to gly-gly (-△-) and *Se*-Bzl-L-SeCys-Gly-OMe (-○-) were determined at different pHs with 50 mM sodium acetate buffer (pH 4-5.5), 50 mM potassium phosphate buffer (pH 5.5-8), 50 mM Tris-HCl (pH 8-9), 50 mM H_3BO_3-NaOH (pH 9-10), and 50 mM H_3BO_3-Na_2CO_3 (pH 10-11).

Optimum pH for enzymatic synthesis of *Se*-Bzl-glutaselenone monomethyl ester was 6.2 (Fig. 5), when L-glutamine and *Se*-Bzl-L-SeCys-Gly-OMe were used as substrates and incubated with *E. coli* γ-GTP at 37 °C for several hours. The production reached the maximum after 12 hours, and 48 % of the acceptor

was converted to the tripeptide. After 15 hours, the amount of Se-Bzl-glutaselenone monomethyl ester decreased.

Although the optimum pH for transpeptidation was 9 for the *E. coli* γ-GTP, glutaselenone was not obtained at high pHs, and white precipitate was formed in the reaction mixture. ^{1}H-nmr analysis showed that the insoluble precipitate contains no γ-glutamyl moiety (data not shown). Kumagai *et al.* also reported (67) that *S*-Bzl-L-Cys-Gly-OMe was converted to a diketopiperazine derivative, which appeared as white precipitate in the reaction mixture of pH 9.

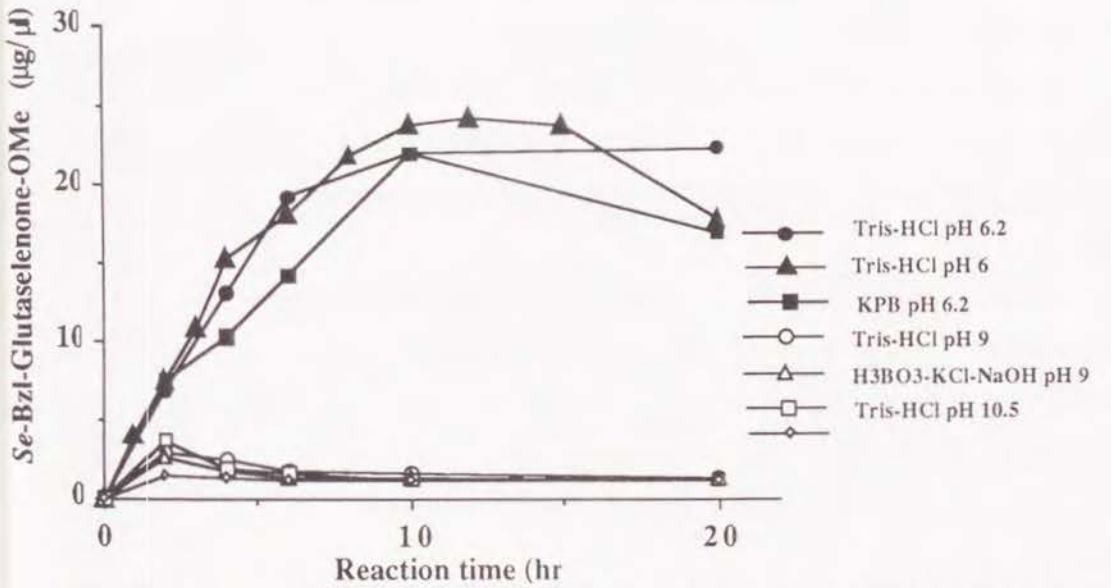

Fig. 5. Effect of pH on synthesis of *Se*-Bzl-Glutaselenone-OMe. The reaction mixture (0.5 ml) containing 100 mM L-glutamine, 100 mM L-*Se*-Bzl-SeCys-Gly-OMe and various buffer was incubated at 37 °C. An aliquot of 20 μl was taken from each reaction mixture at the times indicated, and analyzed by HPLC.

Discussion

γ-Glutamyl amino acids and peptides can be synthesized both chemically and enzymatically, and each method has advantages and disadvantages.

In chemical synthesis of γ-glutamyl amino acids and peptides, introduction of protecting groups to α-amino and α-carboxyl groups of glutamic acid is required before the formation of γ-glutamyl peptide bond. The preparation of the protected form of glutamic acid involves many steps, and requires expensive reagents, and the protecting groups must be removed subsequently.

The enzymatic synthesis with γ-GTP is simple and one step reaction with commercially available γ-glutamyl compounds such as γ–glutamyl-*p*-nitroanilide, glutathione and L- and D-glutamine.

γ-Glutamyl-L-3,4-dihydrophenylalanine (γ-glu-DOPA) was prepared with γ-GTPs from sheep kidney (72), *Proteus mirabilis* (64) and *E. coli* K-12 (69). γ-Glu-L-tyrosine (66) and glutathione (67) were also produced with *E. coli* γ-GTP.

Among various γ-GTPs thus far reported, the γ-GTP from *E. coli* K-12 is most useful for synthesis of γ-glutamyl compounds. The *E. coli* γ-GTP is readily obtained by effective expression of the cloned *ggt* gene, and a rapid purification method (68). The enzyme can utilize L-glutamine as an efficient γ-glutamyl donor. Mammalian γ-GTPs act on L-glutamine to cause hydrolysis (71).

Although the present enzymatic method for the synthesis of γ-glu-peptides is simple, it requires separation of the product from substrates by means of preparative HPLC.

The production of γ-glutamyl peptide with γ-GTP may be accompanied by various side reactions. Autotranspeptidation (71) is a reaction in which γ–glu moiety is transferred to γ-glutamyl donors and products, and formation of γ-glu-glutathione (64), γ-glu-glutamine (65) and γ-glu-γ-glu-L-tyrosine-OMe (66) have been reported. Such a side reaction occurs when the concentration of

γ-glutamyl donor was excessive over that of γ-glutamyl acceptor. In the present enzymatic synthesis with L-Gln and *Se*-Bzl-L-SeCys-Gly-OMe, 100 mM γ-glutamyl donor and acceptor were used to prevent autotranspeptidation.

However, another side reaction occurred to the γ-glutamyl acceptor, and decreased the yield significantly. Formation of diketopiperazine derivatives from the dipeptide acceptor seemed to be catalyzed by γ-GTP, because the white precipitate was not formed in the absence of the enzyme. The cyclization of the acceptor dipeptide seemed to be promoted at high pH, 8-9, and suppressed at lower pH, e.g. pH 6. In the present investigation, the side reaction was minimized by reaction low at pH, for the optimum incubation time.

Summary

The author described a method for synthesis of LL-glutaselenone with γ-glutamyltranspeptidase (γ-GTP). Substrate specificity of γ-GTPs from *E. coli* and bovine kidney and the effect of pH on the enzymatic reaction were examined.

The *E. coli* γ-GTP utilized L-glutamine as a γ-glutamyl donor and *Se*-Bzl-L-SeCys-Gly-OMe as an acceptor, whereas the bovine kidney enzyme catalyzed hydrolysis of L-glutamine, but only little transpeptidation from L-glutamine.

Therefore, the *E. coli* enzyme was chosen for enzymatic synthesis of LL-glutaselenone derivative. The reaction was carried out at pH 6.2 to avoid the formation of a by-product, a diketopiperazine derivative. The production reached the maximum after 12 hours and the yield was 48%.

The enzymatic method is advantageous over a chemical method because L-glutamine is used as a substrate: derivatization and deprotection of γ-glutamyl moiety are not necessary, although the reaction was carried out under the optimum conditions.

Chapter III.

Glutathione Peroxidase-like Activity of Glutaselenone, a Selenium Analog of Glutathione

Glutathione peroxidase (glutathione: hydrogen peroxide oxidoreductase, EC 1.11.1.9) is a selenoenzyme which catalyzes reductive decomposition of H_2O_2 and organic hydroperoxides with concomitant oxidation of glutathione (17, 73, 74). The enzyme plays a central role in the detoxification of peroxides in various tissues such as liver (75), kidney (75), erythrocyte, and lens of eyes (76), and protects membrane and other tissues from oxidative damage (77).

Glutathione peroxidase is composed of four identical subunits, and each subunit contains one selenocysteine residue at the active site. Based on the X-ray crystal structure with 0.2-nm resolution , as well as biochemical data, a catalytic cycle that involves selenol (Enz-SeH), selenenate (Enz-SeOH), and selenosulfide (Enz-Se-SG) forms of selenocysteine residue is proposed (Fig. 6) (78).

Glutaselenone, a selenium analog of glutathione (79), shows glutathione peroxidase-like activity, and it serves as a model for the selenoenzyme, glutathione peroxidase. Glutaselenone also serves as a substrate to glutathione reductase, and the product, glutaselenone selenolate (GSe^-), . In this chapter, these two biochemical features of glutaselenone are described with emphasis of its glutathione peroxidase-like activity.

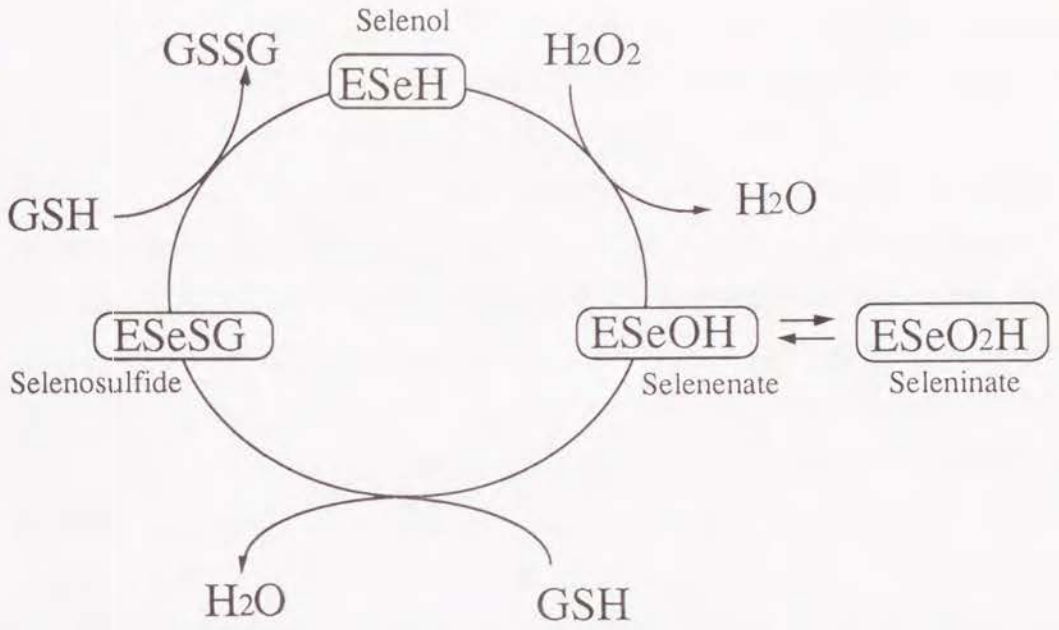

Fig. 6. Reaction mechanism proposed for glutathione peroxidase

Materials.

Reduced and oxidized forms of glutathione , hydrogen peroxide, NADPH and *o*-phthalaldehyde were purchased from Nacalai Tesque (Kyoto, Japan). Glutathione reductase was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Four diastereomers of glutaselenone, LL-, DL-, LD-, and DD-isomers were synthesized as described in chapter I.

Preparation of glutaselenone selenosulfide (GSeSG).

Aqueous solution (1 ml) containing 100 mM glutathione (GSH), 10 mM glutaselenone (GSeSeG) and 40 mM hydrogen peroxide was incubated at 37°C for 30 min. Selenosulfide (GSeSG) was isolated by preparative HPLC as described below. (2 mg, yield 30%). Atomic absorption: 1 pmol Se / pmol
^{1}H-nmr (200MHz, D_2O) : δ 2.1 (2H, q, γ-Glu-β–CH_2),
2.5 (2H, t, γ-Glu-γ-CH_2), 3.2 (2H, m, SeCys, Cys-β-CH_2),

3.9 (1H, t, γ-Glu-α-CH), and 4.0 (2H, s, Gly-CH_2);
UV spectrum: λmax 205 nm (ε = 7800), λmax 270 nm (ε= 350).

Preparation of glutaselenone seleninic acid ($GSeO_2H$).

Aqueous solution (1 ml) containing 10 mg of glutaselenone diselenide (28.3 μmol) and hydrogen peroxide (113 μmol) was incubated at 37°C for 30 min. Glutaselenone seleninic acid was purified by preparative HPLC as described below (8mg, yield 90%).
^{1}H-nmr (200 MHz, D_2O): δ 2.1(2H, q, γ-Glu-β-CH_2), 2.5 (2H, t, γ-Glu-γ-CH_2), 3.2 (4H, m, SeCys, Cys-β-CH_2), 3.9 (1H, t, γ-Glu-α-CH), and 5.2 (2H, s, Gly-CH_2); ^{13}C-nmr (50 MHz, D_2O) : δ 25.6, 30.9, 41.3, 49.3, 52.6, 56.8, 172.3, 172.8, 173.0, and 174.4; UV spectrum : λmax 200 nm (ε = 6200), λmax 275 nm (ε=100), λmax 350 nm(ε= 40)

Conditions for analytical and preparative HPLC.

Reduced and oxidized forms of glutathione, and glutaselenone derivatives were analyzed by HPLC under the following conditions. Waters HPLC pump system equipped with a 600E system controller, a U6K universal liquid chromatograph injector and a 484 tunable absorbance detector (wavelength fixed at 210 nm) were used. The solvent system, 2.5% acetonitrile (v/v) and 0.1% trifluoroacetic acid (v/v) in water, was used at a flow rate of 1 ml/min for analytical HPLC. For preparative HPLC, the flow rate was 5 ml/min.

A Cosmosil C18 (4.6 mm x 15 cm) column was used for analytical HPLC. Glutaselenone and its derivatives were identified by co-chromatography with standard reagents: GSH, 3 min; GSSG, 6 min; GSeSeG, 11 min; GSeSG, 8 min.

A ULTRON C18 column (1.0 x 25 cm) was used for isolation of glutaselenone selenosulfide (GSeSG). The retention times of glutaselenone and its derivatives were: GSH, 4 min; GSSG, 6 min; GSeSeG, 10 min; GSeSG, 7

min. A μBondaspack C18 column (1.9 x 15 cm) was used for isolation of glutaselenone seleninic acid ($GSeO_2H$), which was eluted at 8 min. Glutaselenone diselenide (GSeSeG) was eluted at 9.4 min.

Glutathione peroxidase-like activity of $GSeO_2H$

A solution (1 ml) containing 0.5 mM $GSeO_2H$ and 3 mM H_2O_2 in 1 ml of 10 mM potassium phosphate buffer (pH 7.0) was incubated at 37°C for 40 min. Reduced form of glutathione (2 mg, 6.5 μmol) was added to the mixture. A 10 μl-aliquot of the mixture was taken every 15 min, and subjected to analytical HPLC.

Glutathione peroxidase-like activity of GSeSeG

A solution (1 ml) containing 0.7 mM GSeSeG and 6.5 mM GSH in 1 ml of 10 mM potassium phosphate buffer (pH 7.0) was incubated at 37°C for 40 min. Hydrogen peroxide (final concentration, 3 mM) was added to the mixture. A 10 μl-aliquot of the mixture was taken every 15 min and subjected to analytical HPLC.

Glutathione peroxidase-like activity of selenosulfide

A solution containing reduced form of glutathione (100 μM) and LL-glutaselenone selenosulfide (50 μM) was incubated at 37°C for 60 min. Hydrogen peroxide was added (final concentration, 50 μM), and a 10 μl-aliquot of the mixture was taken every 15 min and subjected to analytical HPLC.

Glutathione reductase activity on glutaselenone diastereomers

Glutathione reductase activity was determined by the procedure of Carlberg *et al.* (80) with some modifications. The assay mixture (0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA,

glutaselenone (24 μM), and 0.2 mM NADPH. The reaction was started by addition of glutathione reductase (0.024 U), and carried out at 37 °C. The rate of NADPH oxidation was measured spectrophotometrically at 340 nm.

Glutathione peroxidase-like activity of glutaselenone diastereomers.

I. GR-coupled assay

Glutathione peroxidase-like activity of glutaselenone diastereomer was determined by the method developed by Little *et al.* (61). The assay mixture (95 μl) contained 50 mM potassium phosphate buffer (pH 7.0), 5 mM EDTA, 1 mM NaN_3, 1 mM GSH, glutathione reductase (0.024 U), 0.16 mM NADPH and 10 μM glutaselenone diselenide (20 μM Se). The reaction was started by addition of 5 μl of 4 mM hydrogen peroxide, and carried out at 37°C. The rate of NADPH oxidation was measured spectrophotometrically at 340 nm. H_2O_2 was replaced by H_2O in a control experiment. Glutaselenone was omitted for a blank experiment.

II. *o*-Phthalaldehyde method

Glutathione peroxidase-like activity of glutaselenone diastereomer was determined by *o*-phthalaldehyde method (Fig. 7) (81). The assay mixture was the same as described above except that NADPH and glutathione reductase were omitted. The reaction was incubated at 37°C, and started by addition of hydrogen peroxide as described in GR-coupled assay. After incubation for 30 sec, 1 min and 2 min, the reaction was terminated by addition of 20 μl of 0.1 N *N*-ethylmaleimide. The mixture was incubated at room temperature for 30 min and mixed with 280 μl of 0.1 N NaOH. A 100 μl-aliquot of the mixture was mixed with 100 μl of *o*-phthalaldehyde solution (1 mg/ml methanol) and 1 ml of 0.1 N NaOH solution, and incubated at room temperature for 15 min.

The fluorescent compound derived form glutathione disulfide (Fig. 7) was

determined by a fluorescent spectrophotometer on excitation at 350 nm and emission at 410 nm. H_2O_2 was replaced by H_2O in a control experiment. Glutaselenone was omitted for a blank experiment.

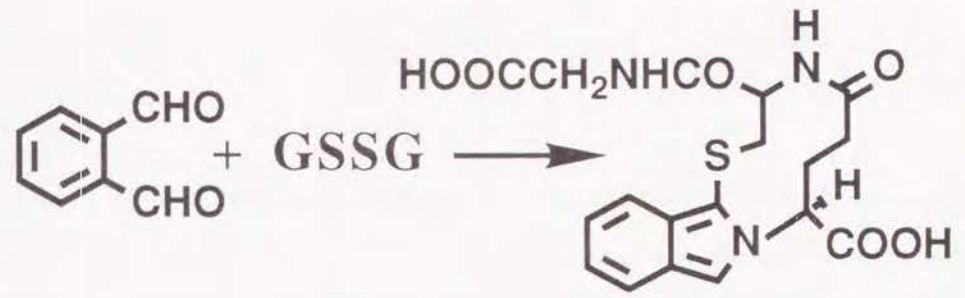

Fig. 7. The principle of *o*-phthalaldehyde method. Glutathione disulfide is derivatized to a fluorescent compound with *o*-phthalaldehyde, and measured on excitation at 350 nm and emission at 410 nm.

Results

Preparation of glutaselenone seleninic acid ($GSeO_2H$) and selenosulfide (GSeSG).

The reaction mechanism proposed for glutathione peroxidase involves various chemical forms of the enzyme-bound selenium (Fig. 6). Some of them are stable and readily isolated, and others are very reactive and difficult to detect even *in situ*.

House *et al.* (82) prepared seleninic acid ($E\text{-}SeO_2H$), selenolate ($E\text{-}Se^-$) and selenosulfide (E-Se-SR) forms of selenosubtilisin, an artificial selenoenzyme that catalyzes glutathione peroxidase reaction, but selenenic acid form (E-SeOH) was not identified (52).

Three possible intermediate forms of glutaselenone that cause the glutathione peroxidase-like activity were prepared; glutaselenone diselenide

(GSeSeG), seleninic acid (GSeO2H) and selenosulfide (GSeSG). Selenolate form (GSe^-) was also prepared by reduction of glutaselenone diselenide with sodium borohydride or dithiothreitol, but it was readily oxidized to diselenide form under the aerobic conditions. Glutaselenone selenenic acid (GSeOH) was not identified. Alkyl-selenenic acids are known to be very unstable chemical species, and only aryl-selenenic acids stabilized by *ortho*-substitution have been characterized (73, 79, 83).

Glutathione peroxidase-like activity of glutaselenone diselenide (GSeSeG) and seleninic acid ($GSeO_2H$).

It was previously reported that selenosulfides are produced from diselenides or seleninates. Yasuda *et al.* reported that selenocystine and glutathione formed a selenosulfide conjugate (SeCys-Se-S-Glutathione) in the presence of hydrogen peroxide (84). Kice *et al.* reported that benzene seleninic acid was converted to a selenosulfide (Ph-Se-S-*t*-Bu) by a reaction with *t*-butyl mercaptan, hydrogen peroxide was not required for the conversion of seleninate to selenosulfide (85).

Glutaselenone diselenide and seleninic acid were converted to selenosulfide (GSeSG), in the presence of glutathione and hydrogen peroxide, and catalyzed glutathione peroxidase-like reaction (Fig. 8). When glutaselenone diselenide was incubated with glutathione (GSH), it was converted gradually to selenosulfide form. The conversion was stimulated by addition of hydrogen peroxide, and completed in 50 min.

Glutaselenone seleninic acid was incubated with hydrogen peroxide, and glutathione was added. Glutaselenone seleninic acid was rapidly converted to selenosulfide in 40 min.

The rate of glutathione disulfide formation by seleninate form was higher than that by diselenide. This is probably because seleninate is more oxidized form than diselenide. These results are in agreement with the previous reports

that ebselene forms a selenosulfide intermediate when it catalyzes glutathione peroxidase-like reaction (86, 87).

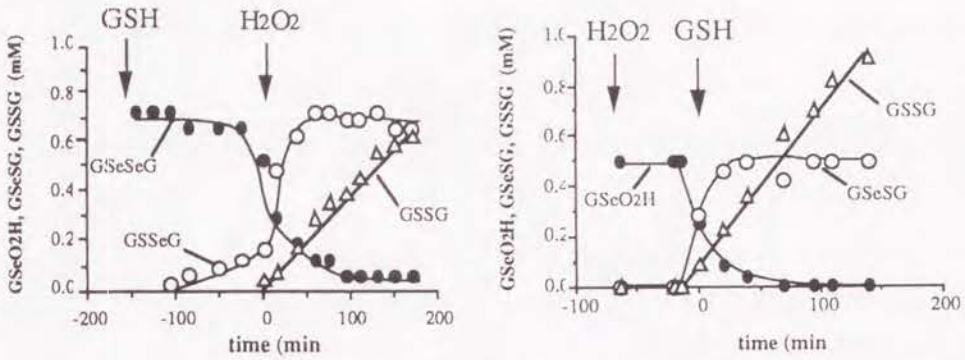

Fig. 8. Treatment of Glutaselenone diselenide and seleninate with GSH / H_2O_2. At the point t=0, glutathione peroxidase-like reaction was started by mixing H_2O_2 or GSH.

Glutathione peroxidase-like activity of glutaselenone selenosulfide (GSeSG).

According to the reaction mechanism proposed for glutathione peroxidase (Fig. 6) (78), nucleophilic attack of glutathione (GSH) on selenosulfide intermediate (Enz-Se-SG) provides glutathione disulfide (GSSG) and selenolate form of the enzyme ($Enz\text{-}Se^-$).

However, glutaselenone selenosulfide and glutathione reacted very slowly, and hydrogen peroxide was required to carry out the reaction (Fig. 9). The concentration of glutaselenone selenosulfide appeared to be constant during the oxidation of glutathione.

Engman *et al.* reported that formation of disulfide from alkyl mercaptan and selenosulfide form of ebselene was considerably faster in the presence of hydrogen peroxide (88).

Glutathione reductase activity on glutaselenone diastereomers.

Glutaselenone diselenide and selenosulfide are selenium analogs of glutathione disulfide, and served as substrates for glutathione reductase . Glutathione reductase activity on glutaselenone diastereomer was in the order of LL-, DL-, LD- and DD-isomers, for diselenide and selenosulfide forms (Table VI).

Table VI. Glutathione reductase activity on GSeSeG and GSeSG

Glutaselenone (24 μM Se)	GSeSeG(diselenide) μM /min	relative rate	GSeSG(selenosulfide) μM/min	relative rate
LL	7.2	1	2.4	1
DL	2.1	0.3	0.56	0.2
LD	0.3	0.04	0.46	0.2
DD	0.3	0.04	0.40	0.1

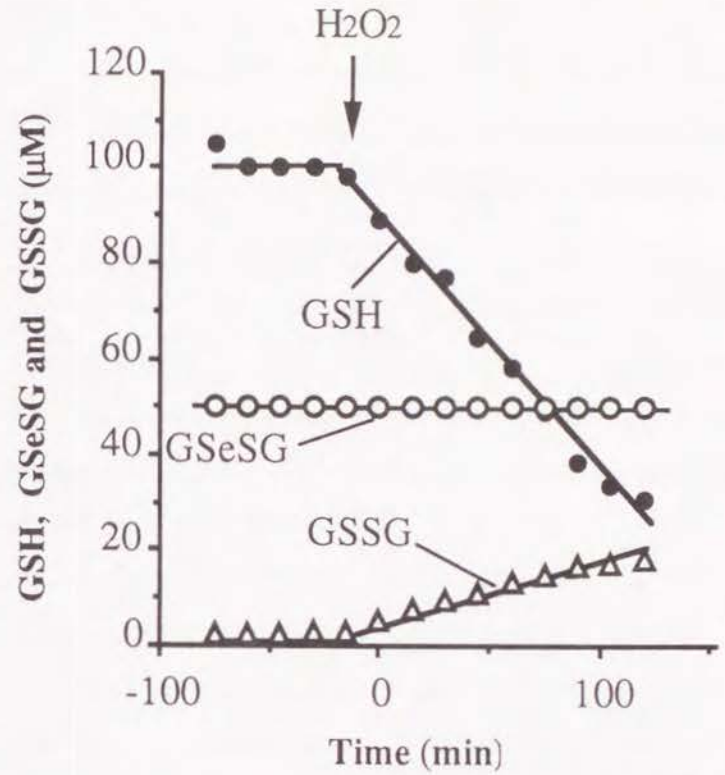

Fig. 9. Treatment of the selenosulfide intermediate with GSH/H_2O_2.
At the point t=0, H_2O_2 was added.

Glutathione peroxidase-like activity of glutaselenone diastereomers.

The glutathione peroxidase-like activity of LL-, DL-, LD-, and DD-glutaselenone was 0.9, 0.3, 0.2, and 0.06 min^{-1}, respectively, by GR-coupled assay. But they showed the same activity, 0.4 min^{-1} by *o*-phthalaldehyde method (Table VII). Glutathione reductase activity on glutaselenone diastereomer (Table VI) was compatible with the glutathione peroxidase-like activity shown by GR-coupled assay. The enzyme probably acted on glutaselenone diselenide and / or the selenosulfide intermediate.

I here suggest a catalytic cycle that involves the action of glutathione reductase (Fig. 10): reductive cleavage of glutaselenone produces selenolate (GSe^-), and it decomposes hydroperoxides to form selenosulfide (GSeSG). The selenosulfide is cleaved by the enzyme and the cycle is completed.

Table VII. Glutathione peroxidase-like activity of glutaselenone diastereomers

Glutaselenone (20 μM Se)	Glutathione Reductase Assay μM GSSG / min	turnover/min	*o*-Phthalaldehyde Assay μM GSSG / min	turnover/min
LL	18.0	0.9	8.7	0.4
DL	5.8	0.3	7.7	0.4
LD	3.0	0.2	8.5	0.4
DD	1.1	0.1	7.7	0.4

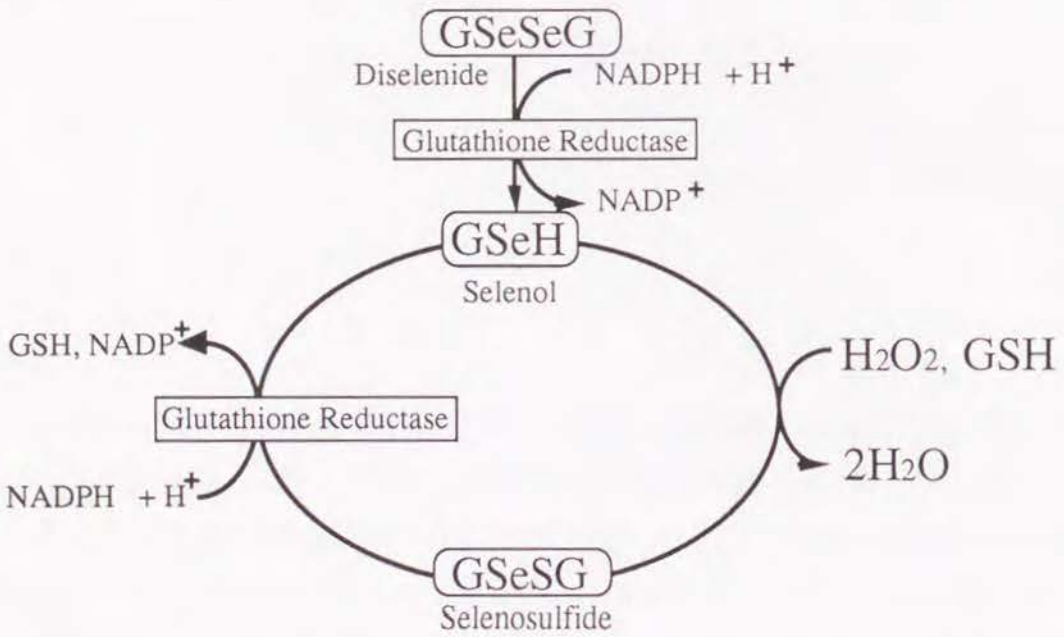

Fig. 10. A proposed mechanism for reductive decomposition of hydrogen peroxide by NADPH.

Discussion

The selenocysteine residue of glutathione peroxidase is able to take diverse chemical forms, but the predominant form that occurs in the enzyme reaction has never been characterized chemically. Glutaselenone was exploited as a model compound for the investigation of chemical reactions that are carried out by the selenocysteine residue in enzyme.

Glutaselenone diselenide and seleninic acid were rapidly converted to selenosulfide form (GSeSG), and catalyzed glutathione peroxidase-like reaction in the form of selenosulfide. Yoshida *et al.* reported that selenosulfides are thermally stable compounds, but they are very reactive toward radicals (89). This is consistent with the result that hydrogen peroxide is required for activation of selenosulfide (Fig. 9). Although any oxidized forms of selenosulfide intermediates were characterized, seleninic acid thiol ester (R-Se(O)-S-R) is the most probable chemical species to be formed by the reaction with selenosulfide and hydrogen peroxide.

Thus, I here suggest a catalytic cycle that involves selenosulfide, seleninic acid thiol ester (R-Se(O)-S-R) and selenenic acid (Fig. 11). This hypothesis is supported in part by previous findings on seleninic acid thiol ester (Ph-Se(O)-S-R). Kice *et al.* reported that benzeneseleninic acid thiol ester (Ph-Se(O)S-*t*-Bu) reacted with thiol (HS-*t*-Bu) to form disulfides (*t*-Bu-S-S-*t*-Bu) and selenosulfide (Ph-Se-S-*t*-Bu) (85, 90).

In this proposed mechanism, seleninate ($GSeO_2H$) and selenolate (GSe^-) are considered to be formed under highly oxidized and reduced conditions, respectively.

The reaction mechanism proposed for glutaselenone may be applicable to study of mechanism of glutathione peroxidase, e.g., selenocysteine residue is in the form of selenosulfide, and catalyzes glutathione peroxide reaction by

rapid formation of seleninate thioester (E-Se(O)-SG) and selenenate (E-SeOH).

However, selenocysteine residue in these selenoprotein may have chemical properties slightly different from that of glutaselenone, since the enzyme-bound selenium is stabilized by hydrogen bonding to amino acid residues in the active site. The crystal structure of glutathione peroxidase shows that the selenolate anion (Enz-Se$^-$) forms hydrogen bonds to Trp148 and Gln70 (91). Selenium in selenosubtilisin has interaction with Asp32, His64, and SeCys221 (82). Therefore, the reaction mechanism proposed for glutaselenone may be altered in detail by contribution of such hydrogen bonding.

When glutathione reductase and NADPH were involved in glutathione peroxidase-like reaction of glutaselenone, reduction of hydrogen peroxide seemed to be supported by oxidation of NADPH. Glutaselenone served to mediate redox potential of NADPH to hydrogen peroxide in a form of selenolate. Since redox potential of NADPH (-0.324 V) is lower than that of glutathione (-0.10 V), it can afford reduction of hydrogen peroxide theoretically. The reaction should be called as NADPH peroxidase-like reaction rather than glutathione peroxidase-like reaction.

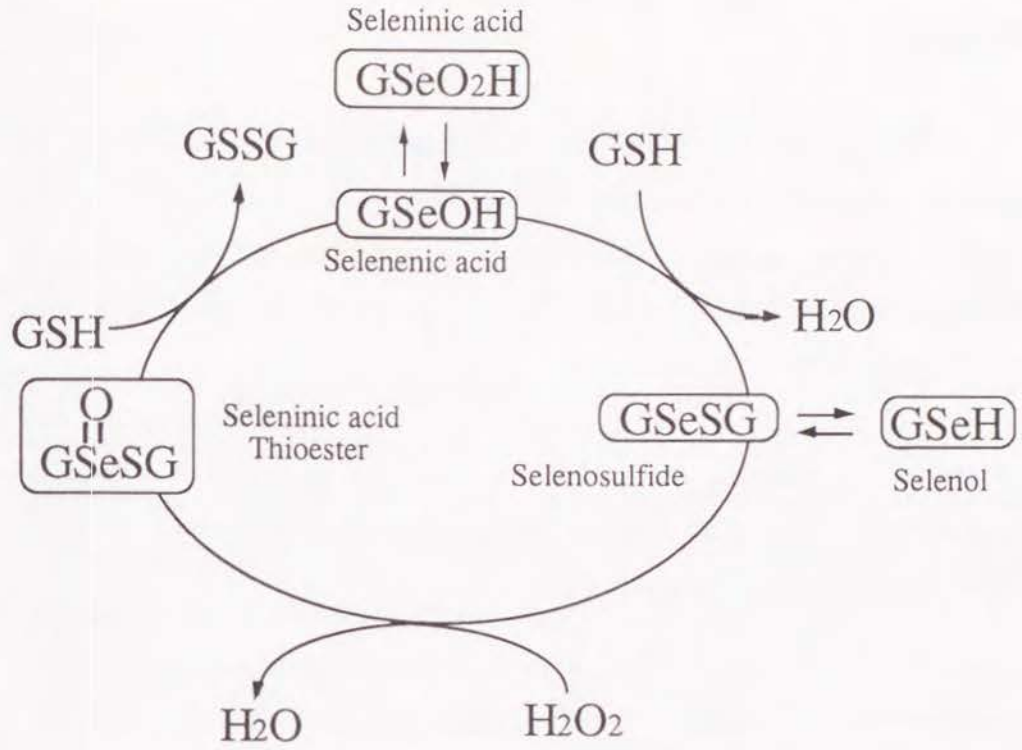

Fig. 11. Proposed mechanism of glutathione peroxidase-like reaction of glutaselenone. Since seleninic acid thioester (GSe(O)SG) and selenenic acid (GSeOH) forms are reactive species, glutaselenone selenosulfide (GSeSG) appears to be the predominant chemical form found in the catalysis.

Summary

Glutaselenone was exploited as a model for enzyme-bound selenium of glutathione peroxidase. Glutaselenone catalyzes glutathione peroxidase-like reaction in a form of selenosulfide (GSeSG). A new catalytic cycle involving a seleninic acid thiol ester (GSe(O)SG) was proposed for the glutathione peroxidase-like reaction of glutaselenone.

Glutaselenone also served as a substrate for glutathione reductase, and transferred redox potential of NADPH to hydrogen peroxide, in a form of selenolate anion. This is an artificial catalytic cycle mediated by a selenium analog of naturally occurring compound.

Chapter IV.

Synthesis and Glutathione Peroxidase-like Activity of a Novel Peptide, SeCys-Gly-Pro-Cys

Thioredoxin is a ubiquitous protein which is involved in a variety of biochemical redox reactions, such as reduction of inorganic sulfate and methionine sulfoxide (92, 93) in microorganisms and biosynthesis of deoxyribonucleotides (94) in all living cells. It also plays an important regulatory role in photosynthesis (95).

All the prokaryotic and eukaryotic thioredoxins contain a conserved sequence, -Cys-Gly-Pro-Cys-, at the active site (96-99). The active site forms a 14-membered disulfide loop in the oxidized form, and reduced to a dithiol by action of an FAD-containing enzyme, thioredoxin reductase (Fig. 12.).

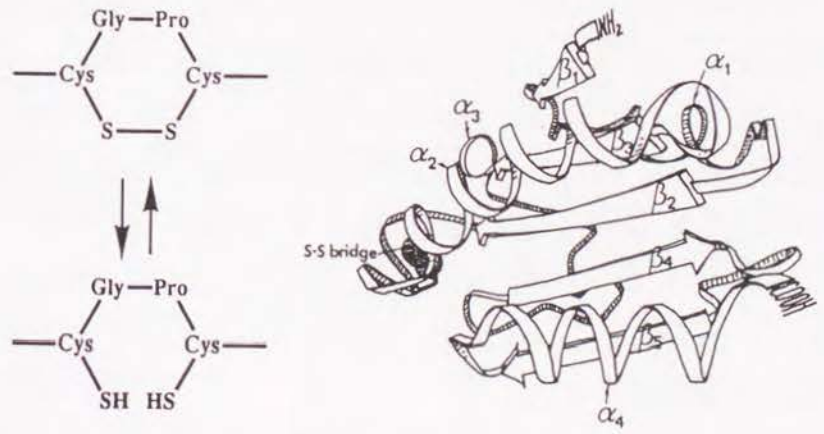

Fig. 12. Structure of thioredoxin and the active site sequence

Two peptide models for the thioredoxin active site, Boc-Trp-Cys-Gly-Pro-Cys-NHMe and Boc-Cys-Gly-Pro-Cys-NHMe, were synthesized chemically (100, 101). The models were oxidized to form an intramolecular disulfide bond, and the resulting β-turn conformation was characterized.

I have shown in chapter III that glutaselenone selenosulfide (GSeSG) is a key intermediate in glutathione peroxidase-like reaction. The active site sequence of thioredoxin is expected to serve as a template for intramolecular selenosulfide bond formation. I here describe the synthesis and characterization of a tetrapeptide, SeCys-Gly-Pro-Cys, and its glutathione peroxidase-like activity.

Materials and Methods

Materials.

N-(*p*-Methoxybenzyloxycarbonyl)-*Se*-(*p*-methoxybenzyl)-L-selenocysteine (PMZ-SeCys(Se-MBz)-OH) was synthesized from *Se*-(*p*-methoxybenzyl)-L-selenocysteine and *p*-methoxybenzyl azidoformate (95).

S-Benzyl-cysteine was purchased from Wako Pure Chemicals, and derivatized to *S*-benzyl-L-cysteine benzyl ester with benzyl bromide (102).

N-*t*-Butyloxycarbonyl-L-proline (Boc-Pro) and *N*-*t*-butyloxycarbonyl-glycine (Boc-Gly) were synthesized from Boc-SDP and L-proline or glycine (103). Reduced and oxidized forms of glutathione, hydrogen peroxide, *o*-phthalaldehyde, *N*-ethylmaleimide, sodium hydroxide, ethylenediaminetetraacetic acid (EDTA) and sodium azide were purchased form Nacalai Tesque (Kyoto, Japan). Diphenyldiselenide was from Aldrich (98%). Other reagents were of analytical grade.

Analytical Methods.

Melting point was measured with a Micro Melting Point Determinational

Apparatus TYPE MM-2 (Shimadzu, Kyoto, Japan); optical rotations with Perkin-Elmer 241 (Perkin-Elmer and Co, Ueberlingen); absorption spectra with a Shimadzu UV-260 spectrophotometer; ^{1}H-nmr spectra with a VXR-200 (Varian); mass spectra with JMS-DX 300 (Jeol). Elemental analysis was done with a Yanaco CHN corder MT-3 (Yanagimoto).

^{1}H,^{1}H-COSY spectrum of SeCys-Gly-Pro-Cys.

SeCys-Gly-Pro-Cys (16 mg; 0.035 mmol) was dissolved in 10 mM potassium phosphate buffer (pH 7.0) in 99.8% D_2O (0.6 ml), and mixed with sodium borohydride (2 mg; 0.05 mmol). The solution was transferred to a NMR tube and air was flashed with a stream of nitrogen gas. ^{1}H,^{1}H-COSY spectrum was recorded on a Varian VXR-200 spectrophotometer at ambient temperature.

Determination of Glutathione Peroxidase-like Activity.

Glutathione peroxidase-like activity was determined by *o*-phthal-aldehyde method. The assay mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM GSH, 5 mM EDTA, 1 mM NaN_3 and organoselenium compounds tested at the selenium concentration of 50 μM.

The reaction was initiated by addition of hydrogen peroxide (final concentration, 0.2 mM), and the reaction mixture was incubated at 37°C.

A 100 μl-aliquot of the reaction mixture was taken every 10 min and mixed with 20 μl of 0.1 N *N*-ethylmaleimide. The mixture was kept at room temperature for 30 min, and mixed with 280 μl of 0.1 N NaOH. An aliquot of 100 μl of the resulting mixture was added to the mixture with 100 μl of *o*-phthalaldehyde solution (1 mg/ml methanol) and 1 ml of 0.1 N NaOH, and the

mixture was incubated at room temperature for 15 min. Fluorescence of the mixture was measured by a fluorescence spectro-photometer by excitation on 350 nm and emission at 410 nm. The rate of production of GSSG was plotted against incubation time. H_2O_2 was replaced by H_2O in a control experiment. Glutaselenone was omitted for a blank experiment.

Results

_N_-t-Butyloxycarbonyl-L-prolyl-_S_-benzyl-L-cysteine benzyl ester (Boc-Pro-Cys(S-Bz)-OBz).

S-Benzyl-L-cysteine benzyl ester (4.6 g; 14 mmol) and triethylamine (2 ml) dissolved in dimethylformamide (28 ml) were added to a solution of *N-t*-butyloxycarbonyl-L-proline (2.06 g; 9.6 mmol), *N,N'*-dicyclohexylcarbodi-imide (2.3 g), and 1-hydroxybenzotriazole (1.5 g) in dimethylformamide (40 ml) at 0°C. After 20 hours, the mixture was filtered and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (100 ml), and washed successively with 5% citric acid (10 ml, twice), 5% sodium bicarbonate (10 ml, twice), and saturated sodium chloride solution (10 ml, twice). The ethyl acetate layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The product was crystallized from ethyl acetate and ether. (Yield 3.5 g; 7.0 mmol), mp, 84°C, $[\alpha]^{25}_{D}$,-86°(c=0.5, acetone), EI-MS (m/z), 498[M^+], Found: C, 65.04%; H, 7.06%; N, 5.49%,
$C_{27}H_{34}N_2O_5S$ requires C, 65.03%; H, 6.87%; N, 5.62%,
^{1}H-nmr (200 MHz, $CDCl_3$); δ1.4 (9H, s, Boc), 1.6 (2H, br, Pro-γ-CH_2),
1.9-2.2 (2H, b, Pro-β-CH_2), 2.8 (2H, m, Cys-β-CH_2), 3.4 (2H, b, Pro-γ-CH_2),
3.6 (2H, s, S-CH_2-Ph), 4.3 (1H, m, Pro-α-CH), 4.7 (1H, m, Cys-α-CH),
5.1 (2H, s, Cys-OCH_2-Ph), 7.2 (5H, s, Benzyl), and 7.3 (5H, s, Benzyl)

_N_-t-Butyloxycarbonyl-glycyl-L-prolyl-_S_-benzyl-L-cysteine benzyl ester (Boc-Gly-Pro-Cys(S-Bz)-OBz).

N-t-Butyloxycarbonyl-L-prolyl-*S*-benzyl-L-cysteine benzyl ester (3.5 g, 7 mmol) was treated with 4 N HCl in ethyl acetate (20 ml) for 30 min at room temperature, and solvent was removed by evaporation. The residual gum was dissolved in a mixture of dimethylformamide (14 ml) and triethylamine (1 ml) to give a solution of the amine component.

A solution containing *N-t*-butyloxycarbonyl-glycine (2.5 g, 14 mmol), 1-hydroxybenzotriazole (2.1 g) and dicyclohexylcarbodiimide (3.22 g) dissolved in dimethylformamide (56 ml) was mixed with the solution of amine component, and stirred at 4°C for four days. The mixture was filtered and evaporated under reduced pressure to give a white precipitate. The precipitate was dissolved in ethyl acetate (100 ml), and washed as described above.

Ethyl acetate layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation to give a pale yellow residue.

The product, Boc-Gly-Pro-Cys(S-Bz)-OBz, was crystallized from ethyl acetate at -20°C. (Yield 2 g; 3.5 mmol), mp, 65°C,
$[\alpha]^{25}_{D}$,-89° (c=1.0, acetone), FAB-MS (m/z), 556 [M+1],
^{1}H-nmr (200 MHz, $CDCl_3$): δ1.4 (9H, s, Boc), 1.6-2.2 (4H, br, Pro-β,γ-C_2H_4),
2.8 (2H,dq,Cys-CH_2), 3.4 (2H,m,Pro-δ-CH_2), 3.6 (2H, s, S-CH_2-Ph), 3.9 (2H, dd, Gly-CH_2), 4.6 (1H, d, Pro-α-CH), 4.7 (1H, m, Cys-α-CH), 5.1 (2H, s, Cys-OCH2-Ph), 7.2 (5H, s, Benzyl), and 7.3 (5H, s, Benzyl)

_N_-(_p_-Methoxybenzyloxycarbonyl)-_Se_-(_p_-methoxybenzyl)-L-selenocysteinyl-glycyl-L-prolyl-_S_-benzyl-cysteine benzyl ester (PMZ-SeCys(Se-PMBz)-Gly-Pro-Cys(S-Bz)-OBz).

N-*t*-Butyloxycarbonyl-glycyl-L-prolyl-*S*-benzyl-cysteine benzyl ester (2 g, 3.5 mmol) was treated with 4 N HCl in ethyl acetate (20 ml) for 30 min at room temperature, and the solvent was evaporated. The residue was dissolved in a mixture of dimethylformamide (7 ml) and triethylamine (0.5 ml) to give a solution of the amine component. The amine component solution was added to a mixture of *N*-(*p*-methoxybenzyloxycarbonyl)-*Se*-(*p*-methoxybenzyl)-selenocysteine (2.3 g, 5.2 mmol), 1-hydroxybenzo-triazole (0.8 g), and dicyclohexylcarbodiimide (1.2 g) dissolved in dimethyl-formamide (10 ml), and stirred at 4°C for four days. The reaction mixture was filtered and the solvent was removed by evaporation. White residue was dissolved in ethyl acetate (100 ml), and washed as described above. The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated to give white solid. (Yield 3.0 g; 3.3 mmol) mp, 120°C, $[a]^{25}_{D}$,-70.8° (c=0.4, chloroform),
Found: C, 58.78%; H, 5.63%; N, 6.24%.
$C_{44}H_{50}N_4O_9SeS$ requires C, 59.39%; H, 5.66%; N, 6.24%,
^{1}H-nmr (200 MHz, $CDCl_3$): δ2.0 (3H,b,Pro-β-CH,γ-CH_2), 2.3 (1H,b,Pro-β-CH), 2.9 (4H, m, Cys-β-CH2, SeCys-β-CH_2), 3.5 (2H, m, Pro-δ-CH_2), 3.6 (2H, s, S-CH_2-Ph), 3.71 (2H, s, Se-CH_2-Ph), 3.75 (3H, s, $BzOCH_3$), 3.77 (3H, s, $BzOCH_3$), 4.0 (2H, b, Gly-CH_2), 4.4 (1H,m,SeCys-α-CH), 4.6 (1H, d, Pro-α-CH), 4.8 (1H, m, Cys-α-CH), 5.0 (2H, dd, Cys-OCH_2-Ph), 5.1 (2H, s, PMZ), 7.2 (4H, dd, Benzyl), and 7.3 (14H, m, Benzyl)

L-Selenocysteinyl-glycyl-L-prolyl-L-cysteine.

Liquid ammonia (30 ml) distilled over sodium metal under nitrogen gas was introduced to the fully protected tetrapeptide (1 g, 1.1 mmol). Freshly cut sodium metal was added in a very small piece to the magnetically stirred mixture at -33°C until the blue color persists for 10 min. The mixture was decolorized with a little solid ammonium sulfate, then the ammonia was evaporated to dryness under a stream of nitrogen gas. The residue was

dissolved in distilled water (30 ml) and desalted by Micro Acilyzer S1 (Asahi Kasei, Kawasaki, Japan), then lyophilized to give yellow hygroscopic powder. (Yield 0.3 g; 0.65 mmol). mp, 170-180°C (decomposition); $[\alpha]^{25}_{D}$,-40.0° (c=0.2, H_2O), Found: C, 34.46%; H, 4.87%; N, 12.14%.
$C_{13}H_{23}N_4O_5SeSCl$ requires C, 33.81%; H, 5.02%; N, 12.13%,
UV spectrum: λmax 200 nm (ε=12000), λmax 310 nm (ε=620)
Fluorescence: Excitation 310 nm, Emission 390 nm and 620 nm
^{1}H-nmr (200 MHz, D_2O): δ 1.9–2.0 (3H, b, Pro-β-CH, γ–CH_2),
2.1 (1H, b, Pro-β-CH), 2.6-2.8 (4H, m, Cys-β-CH_2, SeCys-β-CH_2),
3.2 (1H, m, Pro-δ-CH), 3.5 (2H, m, Pro-δ-CH, SeCys-α-CH), 3.6 (1H, b, Gly-α-CH), 4.0 (1H, b, Gly-α-CH), and 4.2 (2H,b, Cys-α-CH, Pro-α-CH)

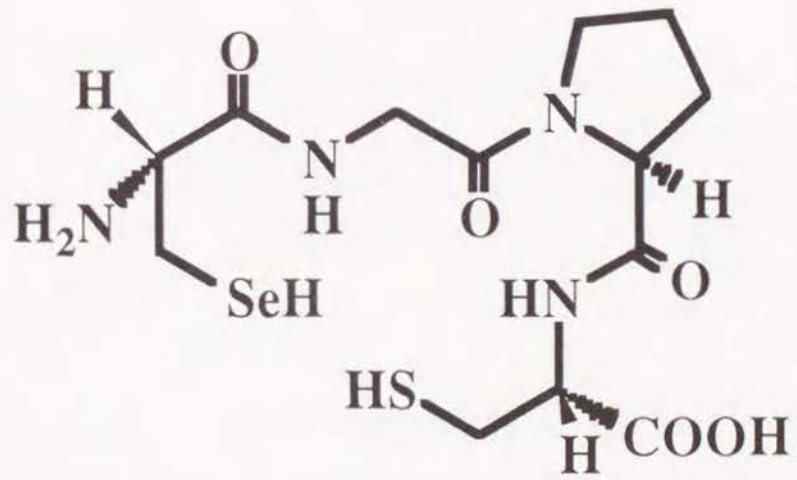

Fig. 13. Structure of SeCys-Gly-Pro-Cys

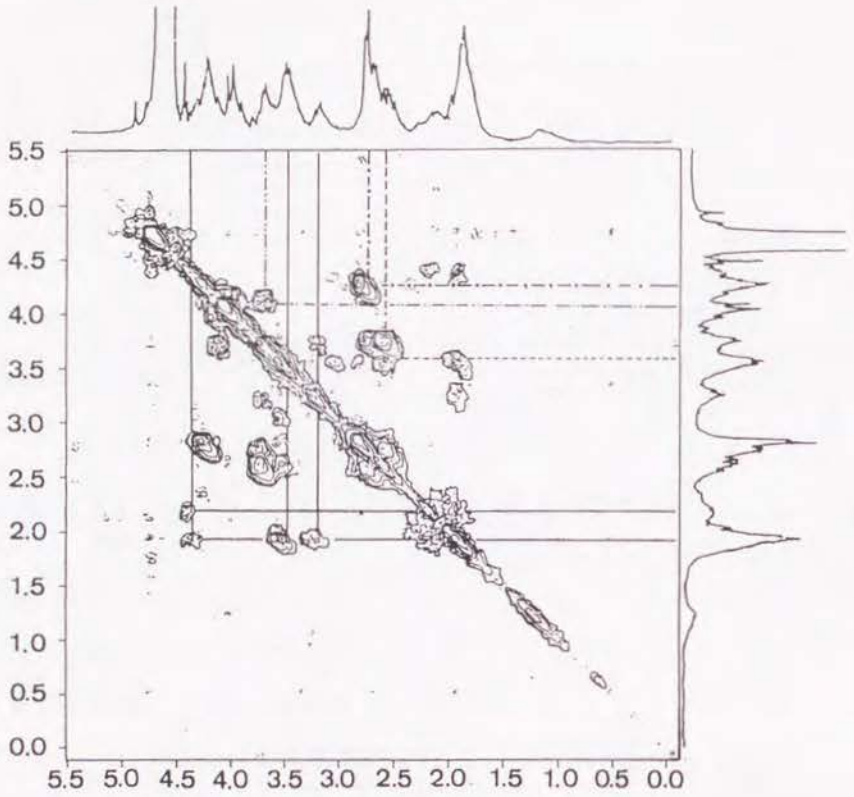

Fig. 14. $^{1}H,^{1}H$-COSY spectrum of SeCys-Gly-Pro-Cys.
Four spin systems are assigned to the constitutive amino acids; selenocysteine (- - - -), glycine (— · · —), proline (——) and cysteine (— · —).

$^{1}H,^{1}H$-COSY Spectrum of SeCys-Gly-Pro-Cys.

The spin systems of the constitutive amino acids were assigned by $^{1}H,^{1}H$-COSY. Since the sample was dissolved in D_2O, amide protons were not observed. Although many of the signals were overlapped with each other, characteristic spin systems were assigned to the constitutive amino acids.

The characteristic spin system, δ 4.1– (δ 1.9, 2.1) – δ 1.9 – (δ3.2, 3.5), was assigned to the proline residue. $^{1}H,^{1}H$-COSY spectrum of L-proline is δ 4.3 (α) – δ 1.9, 2.3 (β) – δ 1.9 (γ) – δ3.3 (δ).

The two protons at δ 3.7 and δ 4.1 showed correlation, and were assigned to glycine residue. Glycine shows a sharp singlet at δ 4.0, and the signal splitting may derive from conformational constraint on glycine.

The complicated signal overlap at δ 2.6-2.8 was assigned to Cys-β-CH_2 and SeCys-β-CH_2. Sharp signal was assigned to the Cys-β-CH_2 and broad signal was assigned to SeCys-β-CH_2, and α-Protons of SeCys and Cys were assigned to δ 3.5 and 4.2, respectively.

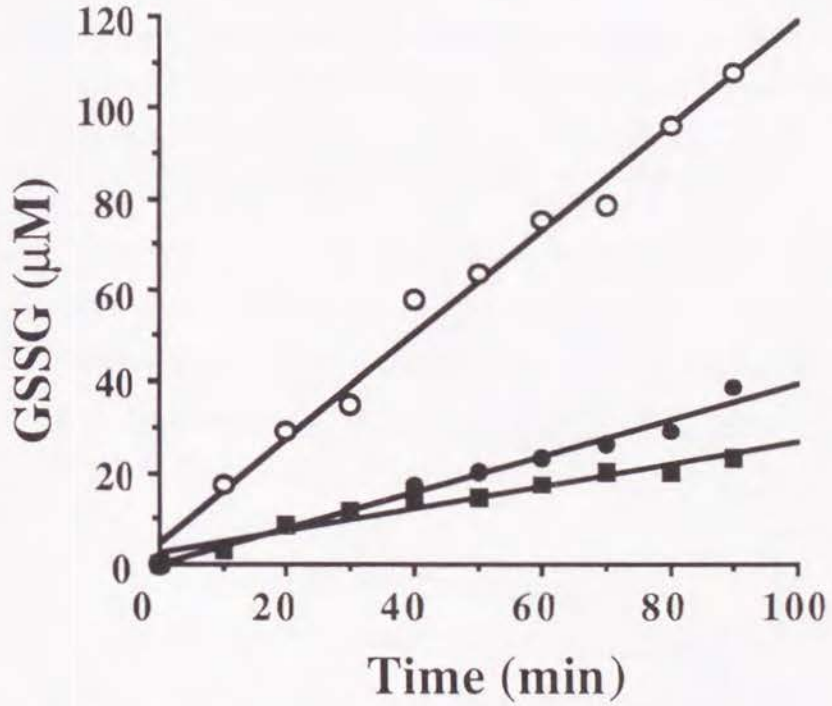

Fig. 15. Glutathione peroxidase-like activity of SeCys-Gly-Pro-Cys (○), glutaselenone (●) and diphenyldiselenide (■). Organoselenium compounds used was at the concentration of 50 μM Se.

Molecular activity of the SeCys-Gly-Pro-Cys.

The tetrapeptide, SeCys-Gly-Pro-Cys, showed glutathione peroxidase-like activity which was almost three times higher than those of LL-glutaselenone and diphenyldiselenide. The three organoselenium compounds were added to the

reaction mixture at a final concentration of 50 μM.

The reaction catalyzed by the tetrapeptide was turned over every 40 min. However, those catalyzed by LL-glutaselenone and diphenyldiselenide was not turned over even after 90 min.

Effect of pH on Glutathione Peroxidase-like Activity.

Glutathione peroxidase-like activity of SeCys-Gly-Pro-Cys and LL-glutaselenone was affected by pH of the assay mixture. However, diphenyl-diselenide showed almost the same activity in the pH range tested (Fig. 16). Rate of decomposition of hydrogen peroxide by a selenol group may be influenced by its degree of dissociation: selenolate anion decomposes peroxides more efficiently than selenol. Selenol groups of selenocysteine-containing peptides are alkyl selenols, whereas that of diphenyldiselenide is an aryl selenol. Effect of pH on the deprotonation is more significant for alkyl selenols than for aryl selenols.

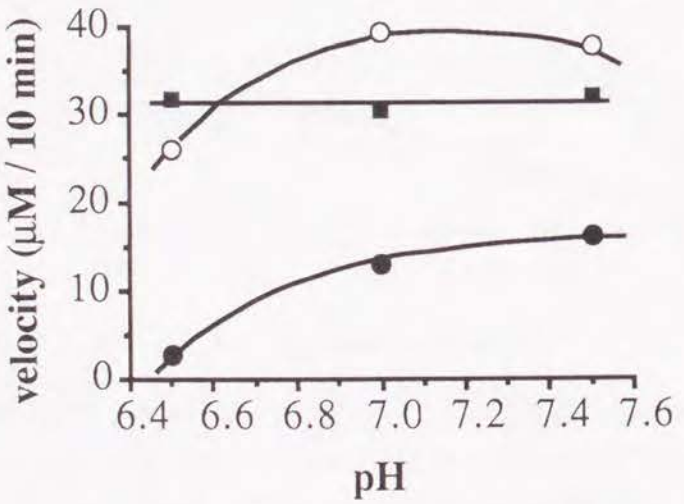

Fig. 16. Effect of pH on glutathione peroxidase-like activity of SeCys-Gly-Pro-Cys (○), glutaselenone (●) and diphenyldiselenide (■). Formation of glutathione disulfide (μM/ 10 min) was determined by *o*-phthalaldehyde method.

Discussion

Synthesis of SeCys-Gly-Pro-Cys.

A selenium analog of the active site sequence of thioredoxin was synthesized by a liquid phase method. The peptide bonds of the tetrapeptide were formed from cysteine to selenocysteine for the smallest loss of selenocysteine.

The selenol group of L-selenocysteine was protected with *p*-methoxybenzyl group. It was also used for chemical synthesis of glutaselenone (79), and proved to be a good protecting group. For introduction and removal are done in a high yield.

α–Amino groups of *S*-benzyl-L-cysteine benzyl ester, L-proline, and glycine were protected with *t*-butyloxycarbonyl (Boc) group, which was selectively removed for peptide elongation. The amino group of L-selenocysteine was protected with *p*-methoxybenzyloxycarbonyl (PMZ) group. All the protecting groups of fully protected tetrapeptide, including the PMZ group, were removed in a final deprotection step with metal sodium in liquid ammonia.

Treatment with metal sodium in liquid ammonia may cause the cleavage of X-proline bonds (X = amino acids), especially in the presence of methanol or moisture (104). Such cleavage was also observed for lysyl-proline bond when *p*-toluenesulfonyl group was removed with Na/NH_3 (48). However, in the previous reports on synthesis of peptide models of thioredoxin active site (100, 101), glycine-proline bond was not cleaved by the deprotection method. In the present synthesis, the fully protected tetrapeptide was crystallized from ethyl acetate and peptide bonds were not damaged by the treatment.

Conformation of SeCys-Gly-Pro-Cys

Formation of disulfide bond between cysteine residues in polypeptide generates compact structures. Intramolecular disulfides occur frequently in peptide hormones, e.g., oxytocin and vasopressin (105), insulin (106), and snake toxins, e.g., siamensis (107). Loops formed by disulfide bond may have considerable flexibility when a large number of amino acids were between the two cysteine residues. However, if the spacer amino acids are only a few, constraints are introduced to the loop structure.

Conformation analysis of synthetic tetrapeptides, Cys-X-Y-Cys (X,Y= Proline, Glycine, Valine and α-aminoisobutyric acid) has been carried out (100, 101, 108-110). Pro-Y segment has a structure of reverse or β-turn (109). X-Pro also has similar reverse or β-turn conformations (110). Boc-Cys-Gly-Pro-Cys-NHMe has an all-*trans* peptide backbone (101) with two consecutive β-turns at Gly-2-Pro-3 and Pro-3-Cys-4.

The conformation of the model peptide is also stabilized by two intramolecular hydrogen bonds (Cys-1-CO and Cys-4-NH, Gly-2-CO and NHMe) (101). Intramolecular hydrogen bond was also identified in Boc-Cys-Pro-Val-Cys-NHMe (108); between the Cys-1-CO and Cys-4-NH .

Thus, the selenium containing peptide, SeCys-Gly-Pro-Cys, is also expected to have a similar consecutive β-turns in the Gly-Pro-Cys segment. A hydrogen bond between SeCys-1-CO and Cys-4-NH may also be formed and stabilize the cyclic conformation. Such a conformational constraint promotes the interaction between the selenol group and the thiol group at both ends of the molecule in the reaction with peroxidase and glutathione.

Summary

A tetrapeptide, Secys-Gly-Pro-Cys, is a selenium analog with the sequence found in the active site of thioredoxin, and forms 14-membered disulfide loop structure. I have synthesized it, and studied its glutathione peroxidase-like activity by *o*-phthalaldehyde assay.

The tetrapeptide showed glutathione peroxidase-like activity three times as high as glutaselenone and diphenyldiselenide. The high catalytic activity is considered to be due to the intramolecular selenosulfide bond formation in the catalysis.

Chapter V

Pharmacological Effects of LL-Glutaselenone

Active oxygen and lipid peroxides serve to induce and develop various diseases, e.g., inflammation, rheumatism, ischemia injury, emphysema, and cataract. For the chemotherapy of these pathological compounds, natural and synthetic antioxidant compounds have been extensively examined. Ebselene is a synthetic organoselenium compound that shows various protective effects against various types of oxidative attack (111), and most of the biological activities are related to its glutathione peroxidase-like activity (112, 113).

Glutaselenone also mimics glutathione peroxidase, and is expected to have pharmacological effects similar to those found for ebselene.

In this chapter, biological effects of LL-glutaselenone investigated in mice are described.

Methods

Phenylquinone Writhing. (114)

A test sample was administered (100 mg/kg, per oral) to a group of three mice one hour before injection of phenylquinone (2 mg/kg, intra peritoneal). The number of writhes per group of animals was counted during 5 to 10 minutes after the phenylquinone injection. When writhing was less than half of that of a vehicle treated group, the test sample was considered to have analgesic activity.

Anti-inflammatory effect (115).

Test substance was administered (100 mg/kg, per oral) to a group of 3 mice, which were not fed overnight. After one hour, carrageenan (0.1 ml, 1 % suspension) was injected in right hind paw. Then, after three hours, more than 30 % inhibition of paw edema formation anti-inflammatory activity was active anti-inflammatory effect.

KCN Hypoxia (116).

Test substance was administered (100 mg/kg, per oral) to a group of 5 mice, 60 minutes before receiving a bolus injection of sub-maximal lethal dose (LD95) of KCN (2.4 mg/kg, inter venous). Survival of more than 3 animals 60 minutes later was considered significant activity.

Chronotropic effect (in vitro) (117).

Spontaneously beating guinea pig right atria were suspended in physiological salt solution at 37°C, and treated with test substance (10 μg/ml). More than 10 % change in atrial rate was considered significant.

Results and Discussion

LL-Glutaselenone showed pharmacological effects at a dosage of 100 mg/kg: analgesic activity, anti-inflammatory effect, and protection from KCN hypoxia. LL-Glutaselenone also made chronotropic response in isolated guinea pig right atria. These biological effects were not observed when the reduced form of glutathione was administered. LL-glutaselenone showed toxicity, when it was administered at a dosage of 1000 mg/kg. (Table VIII)

The analgesic and anti-inflammatory effects are probably related to the

glutathione peroxidase-like activity of glutaselenone. Glutathione peroxidase is present in most organs, and catalyzes reductive decomposition of various hydroperoxides that occur under physiological conditions. However, under the pathological model conditions produced by phenylquinone or carrageenan injection, various active oxygen and lipid peroxides are rapidly produced as chemical mediators for the development of inflammation, and the resulting oxidative force overflows the endogenous antioxidant defense capacity.

Glutathione peroxidase-like activity of glutaselenone probably contributed to decrease the concentration of peroxides, and prevented writhing and edema formation under the pathological model conditions in mice.

Glutathione peroxidase is irreversibly inactivated when treated with potassium cyanide, because selenium of selenocysteine residue is displaced by cyanide (17). Glutaselenone protected mice from KCN hypoxia. Cyanide was probably captured by selenocysteine, in a similar manner to the inactivation of the selenoenzyme.

LL-Glutaselenone decreased atrial rate of guinea pig. Although the mechanism of this biological effect is not clear, it may involve interaction of glutaselenone and some receptor proteins that mediate chronotropic regulation. Chronotropic responses are known to be mediated by several receptors in isolated guinea pig right atria. Selenol group can react with thiol groups in proteins. Glutaselenone may affect certain receptors that regulates atrial rate.

LL-Glutaselenone showed toxicity, when it was administered at a dosage of 1000 mg/kg, ten times as much as the dose that provided beneficial effects. This may also be due to the inhibition of enzymes or receptors by indiscriminate binding of LL-glutaselenone.

Table VIII. Pharmacological effects of LL-glutaselenone

	LL-Glutaselenone			Glutathione			References	
	Dose	Response		Dose	Response		Dose	Response
Phenylquinone Writh	100 mg/kg	77	+	100 mg/kg	31	-	Ibprofen 25 mg/kg	65
KCN Hypoxia	100 mg/kg	60	+	100 mg/kg	0	-	Cinnarizine 30 mg/kg	60
Chronotropic	0.01 mg/ml	-12	+	0.01 mg/ml	-8	-	Mecamylamine 10 mg/kg	-12
Anti-inflammatory	100 mg/kg	38	+	100 mg/kg	0	-	Hydrocortisone 30 mg/kg	31
Autonomic Effect	1000 mg/kg	Died		1000 mg/kg	No Effect			
	333 mg/kg	1/3 Died						
	100 mg/kg	No Effect						

Summary

LL-Glutaselenone showed various biological effects such as analgesic activity, anti-inflammatory effect, and protection from KCN hypoxia in mice. Analgesic activity and anti-inflammatory effects are probably caused by glutathione peroxidase-like activity of glutaselenone. LL-Glutaselenone also made chronotropic response in isolated guinea pig right atria.

LL-glutaselenone showed toxicity when administered at a dosage of 1000 mg/kg, that is ten times more than the dosage that provided those pharmacological effects.

Thus, in spite of the various protective effects observed in mice, the selenocysteine-containing peptide may be too toxic for administration to human body.

Conclusion and Prospect

Selenium compounds have various interesting chemical and biological properties. Life has exploited high reactivity and unique characteristics of organoselenium compounds, especially in the form of selenoprotein. Many of the selenoproteins ever characterized contain a selenocysteine residue, which plays integral roles in the biochemical function of the selenoprotein.

The author described biochemical reactions that involved organo-selenium compounds. These reactions are classified in two categories; enzyme reactions on selenium containing compounds, and glutathione peroxidase-like reaction catalyzed by selenocysteine-containing peptides.

γ-Glutamyltranspeptidase (EC 2.3.2.2) acted on selenium-containing amino acids and peptides as efficiently as on the sulfur analogs. The difference of chemical properties between selenium and sulfur seemed to be decreased, for selenol and thiol groups of γ-glutamyl acceptors were protected by benzyl group.

Glutathione reductase (EC 1.6.4.2) acted on glutaselenone diastereomers with the stereospecificity: LL > DL > LD > DD. The glutathione peroxidase-like activity of the glutaselenone diastereomers was in the order of LL > DL > LD > DD when determined by glutathione reductase-coupled assay, but they showed the same activity by *o*-phthalaldehyde assay. Glutathione reductase seems to participate in catalysis according to the stereospecificity. Thus, the author concluded that reduction of hydrogen peroxide was supported by redox potential of NADPH, and glutaselenone served to mediate the reduction in a

form of selenolate anion.

A selenocysteine-containing peptides, γ-Glu-SeCys-Gly and SeCys-Gly-Pro-Cys, catalyzed glutathione peroxidase-like reaction. The author identified three forms of glutaselenone: diselenide (GSeSeG), seleninic acid ($GSeO_2H$) and selenosulfide (GSeSG). A selenenic acid form (GSeOH) was not identified. Diselenide and seleninic acid were rapidly converted to selenosulfide during the catalysis. The author concluded that the glutaselenone selenosulfide served as a catalyst for glutathione peroxidase-like reaction.

Thioredoxin is a ubiquitous protein involved in a variety of biochemical redox reactions. The protein contains a conserved sequence, Cys-Gly-Pro-Cys, which forms an intramolecular disulfide bond with the peptide backbone in consecutive β-turn conformations. The author synthesized a tetrapeptide, SeCys-Gly-Pro-Cys by a liquid phase method. The glutathione peroxidase-like activity of the tetrapeptide was almost three times more than those of glutaselenone and diphenyldiselenide. The high activity of the tetrapeptide was ascribed to the efficient interaction between selenol group and thiol group at each end of the molecule, that has the similar conformational constraint to that of original sequence.

Glutathione peroxidase mimics are potential remedies for various diseases, such as inflammation, rheumatism, and cataract. Anti-inflammatory and analgesic effects of LL-glutaselenone are explained by the glutathione peroxidase-like activity. Toxic effect of LL-glutaselenone was exerted by the administration of ten times large amount of pharmacological dose. Glutaselenone may have affected critical metabolism by binding to enzymes and receptors indiscriminately.

This thesis described biochemistry of selenoethers, diselenides and selenosulfides, and yet many other classes of selenium compounds are available

as synthetic compounds: triselenides (RSeSeSeR'), selenocyclic compounds, selenocarbonyl compounds (R-CSe-R'), selenamines (RSeNHR') and selenimines (RSeN=R'). These compounds are reactive, and bring about diverse reactions such as nucleophilic substitution, elimination, formation of ring or π–bond, radical quenching, and redox reaction. However, little of these unique chemical properties have been used for biochemical studies.

Thus, the author has a future prospect of selenium biochemistry, which is promising and encouraging in the fusion of organic chemistry and molecular biology.

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